UNIVERSITY OF LIVERPOOL

Regulation of apoptosis of myeloid immune cells: implication for cancer therapy and inflammation

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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I declare that this thesis titled:

“Regulation of apoptosis of myeloid immune cells: implication for cancer therapy and inflammation”

is entirely my own work

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Abstract

Objectives: This project was undertaken to investigate the potential of the human PLB-985 cell line to terminally differentiate, along the myeloid lineage into mature neutrophil-like granulocytes, and to identify the molecular properties of the differentiated PLB-985 cells in comparison with blood neutrophils, in order to establish their usefulness as model of neutrophil differentiation and functions.

Methods: Human neutrophils were isolated from whole blood of healthy, consented, adult donors. The human PLB-985 cells were grown in routine culture media of RPMI 1640 (+10mM L-glutamine). Exponentially growing PLB-985 cells were induced to differentiate into neutrophil-like cells in differentiation culture media of RPMI 1640 (+10mM L-glutamine), supplemented with three differentiation and maturation-inducing agents: ATRA, DMF and sodium pyruvate. Granulocytic differentiation was measured by cell morphology using light microscope, apoptosis was determined by morphology or flow cytometry, cell viability, cell cycle parameters, expression of cell surface receptors and phagocytosis were determined by flow cytometry, oxidative burst activity was measured using flow cytometry and luminol-enhanced chemiluminescence assay, and expression of apoptotic proteins was determined by western blotting.

Results: A modified differentiation protocol and optimisation procedure has been established. Terminally-differentiated, neutrophil-like PLB-985 cells, have been consistently cultured, that resemble mature blood neutrophil morphology, evident by the acquisition of multi-lobed nucleus and granulated cytoplasm, and which had an appreciably extended lifespan. It was discovered that supplementing the differentiation medium with either G-CSF or GM-CSF both improved differentiation and delayed apoptosis of the differentiated cells. These cells underwent cell cycle arrest and progression into apoptosis and they resembled mature blood neutrophils in terms of cell cycle parameters and apoptotic morphology. The differentiation induced expression of anti-apoptotic proteins, Mcl-1 and Bcl-X_L in these cells and their expression levels correlated with cell survival status, whilst Bcl-2 expression was lost. The differentiated PLB-985 cells also displayed phagocytosis and oxidative burst activity, expressed cell surface receptors for CD11b, CD14 and CD16 and expressed strong chemotactic transmigration towards chemoattractants. Finally, JAK inhibitors baricitinib and tofacitinib suppressed the growth, differentiation and viability of the differentiated cells, increased their progression into apoptosis, and down-regulate their responses to G-CSF and GM-CSF signalling. Hence, they may inhibit *in vitro* granulopoiesis, and may have anti-inflammatory activity.

Conclusions: This study has indicated that under appropriate conditions, PLB-985 cells can differentiate terminally into mature neutrophil-like phenotypes that resemble blood neutrophils morphologically and functionally, with an enhanced survival of the mature cells. These differentiated PLB-985 cells may therefore, provide an excellent neutrophil-model system for *in vitro* study of neutrophil differentiation and functions, such as apoptosis regulation.

Publication and presentation

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Abbreviations

aBH3	Activator Bcl-2 homology 3
AKT	Protein kinase B
AMP	Adenosine monophosphate
AML	Acute promyelocytic leukaemia
ANOVA	Analysis of variance
Apaf-1	Apoptotic protease-activating factor-1
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
Bad	B-cell leukaemia-2 associated death promoter protein
Bak	B-cell leukaemia-2 homologous antagonist/killer protein
Bax	B-cell leukaemia-2 associated protein-X
BCA	Bicinchoninic acid
Bcl-2	B-cell leukaemia- 2 protein
Bcl-W	B-cell leukaemia- 2 related protein-W
Bcl-X_L	B-cell lymphoma extra-large protein
Bfl-1/A1	B-cell leukaemia- 2 related protein-A1
BH	B-cell leukaemia- 2 homology domain
Bid	BH3 interacting domain death agonist protein
Bik	B-cell leukaemia- 2 interacting killer protein
Bim	B-cell leukaemia- 2 like protein-11
Bok	B-cell leukaemia- 2 related ovarian killer protein
BSA	Bovine serum albumin
C5	Complement component 5
CAK	CDK-activating kinase
Caspase	Cysteine-aspartic acid protease
CD	Cluster of differentiation
CDC25	Cell division cycle25
CDK	Cyclin-dependent kinase
CGD	Chronic granulomatous disease
COPD	Chronic obstructive pulmonary disease
CR	Complement receptor
CRSP3	Cysteine-rich secretory protein3
dbcAMP	Dibutyryl-cyclic AMP
DAG	Diacylglycerol

DHR	Dihydrorhodamine
DISC	Death-inducing signalling complex
DMARD	Disease-modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dPLB-985	Differentiated PLB-985 cell
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FCS	Foetal calf serum
Fc	Fragment crystallisable region of immunoglobulin
Fcγ	Fragment crystallisable region of IgG
FITC	Fluorescein isothiocyanate
FISH	Fluorescence in situ hybridisation
FMLP	*N*-formyl-methionyl-leucyl-phenylanaline
FPR	Formyl peptide receptor
FS	Forward scatter
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GPI	Glycosylphospatidylinositol
HBSS	Hank’s balanced salt solution
HEPES	4-(2-hydroxyl)-1-piperazineethanesulfonic acid
HMBA	Hexamethylene biacetamide
HMP	Hexose monophosphate
HOCl	Hypochlorous acid
HRP	Horseradish peroxidise
iBH3	Inactivator Bcl-2 homology 3
IC	Immune complexes
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
JAK	Janus kinase

JAM	Junction adhesion molecule
KDa	Kilodalton
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
MAC-1	Macrophage antigen-1
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukaemia-1 protein
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOMP	Mitochondrial outer membrane permeabilisation
MPO	Myeloperoxidase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NSAID	Non-steroidal anti-inflammatory drug
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PECAM	Platelet/endothelial cell adhesion molecule
PI	Propidium iodide
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase c
PMA	Phorbol myristate acetate
PML	Pro-myelocytic leukaemia
PMNL	Polymorphonuclear leukocyte
Puma	P53-upregulated modulator of apoptosis
PVDF	Polyvinylidene Fluoride
RA	Rheumatoid arthritis
RAR-α	Retinoic acid receptor-α
RLU	Relative light unit
RCF	Relative centrifugal force
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAPI	*Staphylococcus* aureus-PI (labelled)

SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
STAT	Signal transducers and activators of transcription
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TM	Transmembrane domain
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRADD	TNF-receptor-associated death domain
TRAF	TNF-receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TPA	Tetradecanoylphorbol-13-acetate
VCAM	Vascular cells adhesion molecule
XIAP	X-linked inhibitor of apoptosis

Chapter 1: Introduction

1.1 Background

Neutrophils or polymorphonuclear leukocytes (PMNs) are key mediators of inflammation and defence against microbial infection. They are the most abundant white blood cells in human blood and the first leukocytes recruited to the site of infection or inflammation. Circulating blood neutrophils are generally-considered to be mature and terminally-differentiated. Therefore, they are short-lived, lack proliferation capacity and presently are impossible to transfect *in vitro* to express exogenous genes or proteins. These properties have made experimental manipulation of neutrophils *ex vivo* challenging, and hindered biochemical studies of their signal activation mechanisms and genetic makeup. In this regard, establishment of cell line models capable of differentiating along the myeloid lineage into mature neutrophil-like granulocytes, which exhibit similar morphological and functional properties to neutrophils are very important tools to enable the manipulation of key genes and proteins, and determine the effects of such modulations on function.

The overall aim of this project was to develop culture conditions that optimised the induction of the human promyelocytic leukaemia PLB-985 cell line to terminally differentiate into mature, neutrophil-like granulocytes, using three differentiation and maturation inducing agents; ATRA, DMF and sodium pyruvate. PLB-985 is a recently-established cell line that has been demonstrated to show greater levels of differentiation and can undergo both granulocyte and monocyte/macrophage differentiation in the presence of appropriate inducing agents. It is currently most commonly-used cell line model for neutrophil differentiation. Previous reports have shown the human PLB-985 cell line as having the potential to differentiate into

neutrophil-like cells under the influence of various chemical agents. However, the efficiency of differentiation reported was very low, and the differentiated cells only partly resembled mature neutrophils (Pivot-Pajot et al., 2010; Ashkenazi & Marks, 2009; Hazan-Eitan et al., 2006; Hiran et al., 2001; Drayson et al., 2001; Martin et al., 1990; Tucker et al., 1987; Souza et al., 1986; Breitman et al., 1980; Collins et al., 1978). In addition, mature neutrophils constitutively undergo apoptosis (Edwards, 1994; Edwards et al., 2004) and so the more efficient the differentiation of PLB-985 cells into mature neutrophils, the greater the extent of apoptosis. This limits the usefulness of these cells as experimental models.

The research focus of this thesis is specifically on the development of a modified differentiation protocol and optimisation procedures to obtain terminally-differentiated PLB-985 cells that functionally and morphologically resemble mature neutrophils, with prominent multiple lobed nuclei, granulated cytoplasm, and with appreciably extended lifespan. Subsequently this research aims to study the molecular and functional properties of the differentiated neutrophil-like PLB-985 cells, in order to establish their usefulness as models of neutrophil differentiation and function.

1.2 Haematopoiesis

Haematopoiesis is the process responsible for production of diverse types of blood cells from haematopoietic stem cells that reside in the bone marrow. (Zhou & Zon, 2001; Orkin, 2000). Blood consists of a number of morphologically and functionally distinct cell types, such as lymphoid cells consisting of B-lymphocytes, T-lymphocytes and NK (natural killer) cells; myeloid cells comprising different subsets of granulocytes (i.e. leukocytes or white blood cells: neutrophils, basophils and eosinophils) and monocytes (precursors of macrophages), macrophages,

erythrocytes (red blood cells), megakaryocytes, mast cells and dendritic cells (DCs) (Iwasaki & Akashi, 2007).

Blood cells have diverse and specialised functions: granulocytes and monocytes play important roles in inflammatory responses and defence against infections. Neutrophils phagocytose micro-organisms such as bacteria and fungi, eosinophils help in destroying parasites, basophils secrete histamines, which upon interactions with cell surface receptors induce many responses such as, increased permeability, enhanced intra-capillary pressure and decreased blood pressure. Monocytes leave the bloodstream and enter tissue to mature into macrophages, and macrophages engulf large organisms and damaged cells. Erythrocytes distribute oxygen bound to haemoglobin to the tissues, while platelets help in blood clotting and repair of damaged vessels. B-lymphocytes produce antibodies, while CD8+ T-lymphocytes kill cells recognised as foreign to the body, including virus-infected and some cancer cells. CD4+ lymphocytes generate chemokines and cytokines that regulate all elements of the immune system (Domen et al., 2006).

In spite of their differences in function, most mature blood cells are considered to be terminally-differentiated cells which do not divide and eventually die within days or weeks after release from the marrow (Edwards, 1994). Therefore, they must be continuously replenished throughout the lifespan. For example, to maintain normal haematopoiesis in an adult human, approximately 20×10^{10} erythrocytes and 16×10^{10} leukocytes must be replenished by proliferation from more primitive cells each day (Bellantuono, 2004). Haematopoietic development is a well-regulated but dynamic process that begins during embryogenesis and continues in the bone marrow throughout adulthood, for sustained production of blood cells (McKercher et al., 1996; Edwards, 1994).

1.2.1 Haematopoietic stem cells and progenitor cells

Haematopoiesis begins with pluripotent stem cells that undergo asymmetric cell division to self-renew and also to give rise to other cell lineages (Moss, 2016). Stem cells have the capacity for self-renewal and can differentiate into multiple cell types, depending on the physiological need (Kondo et al., 2003). They are categorized into two types: pluripotent stem cells which can differentiate into the cells of all three germ layers, namely; endoderm, ectoderm, and mesoderm, and the multipotent stem cells which are lineage-specific and more differentiated, such as neuronal stem cells, hepatic stem cells and hematopoietic stem cells (HSCs). Cells differentiate from stem cells via committed progenitors which are restricted in their developmental potential to a specific lineage. The establishment of the haematopoietic system during embryogenesis follows an ordered hierarchical sequence of developmental activations and specifications from the primitive stem cells, such as formation of hematopoietic stem cells, multipotent progenitors, committed precursors and finally, fully-differentiated, mature cells (Bellantuono, 2004). Although stem cells are rarely found in most tissues, each tissue has arisen from a specific stem cell. The principal function of stem cells in adult tissues is to repair and regenerate the tissue in which they reside (Reya et al., 2001; Kondo et al., 2003).

The continued production of blood cells directly depends on hematopoietic stem cells, which are the ultimate and only source of all blood cells. Under normal situations, these cells divide to produce progenitor cells, which, depending on the tissue, can undergo a few cell divisions and subsequent differentiation steps to generate a complex web of mature cells along specific pathways (Figure 1.1). These events result in continuous production of sufficient numbers of cells of all lineages (Joanna et al., 2001; Akashi et al., 2000).

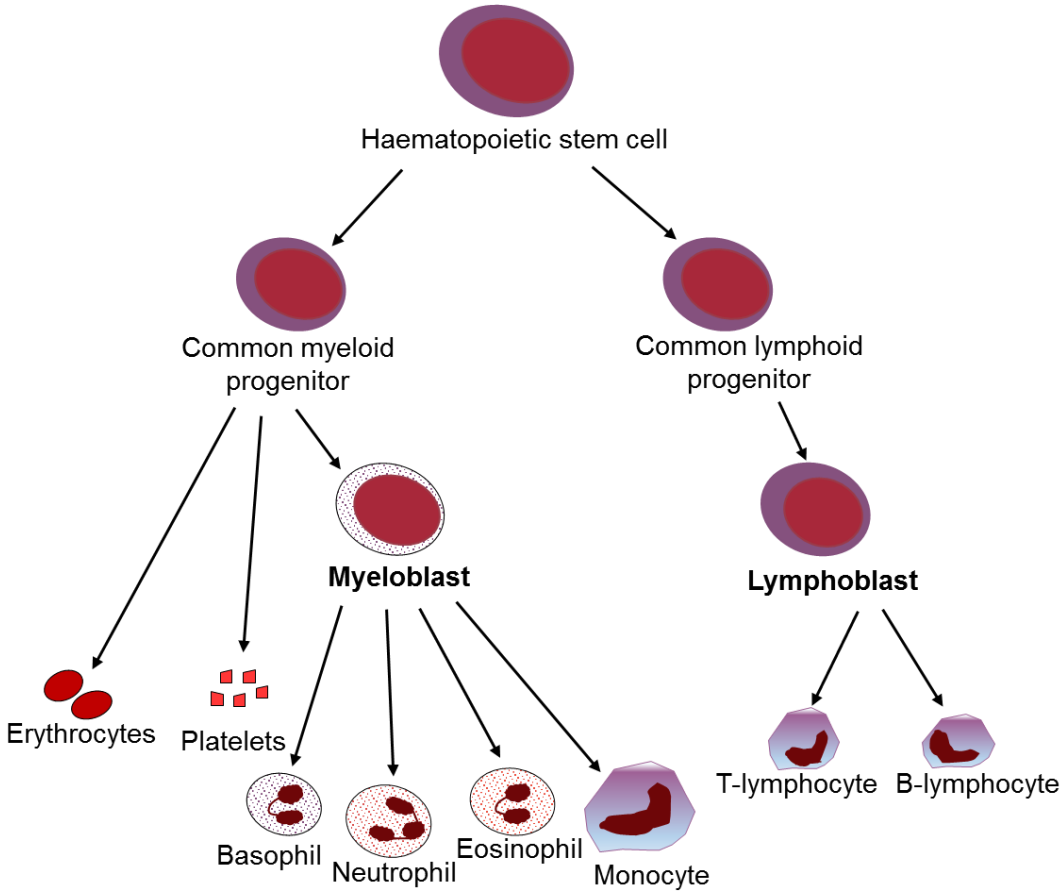


Figure 1.1 Development of different mature blood cells from multipotent haematopoietic stem cells. Stem cells divide into two daughter cells, one retains the characteristics of the original cell for self-renewal, whilst the other one matures into committed haematopoietic precursors, that give rise to either common myeloid progenitors (CMP) or common lymphoid progenitors (CLP). CMP give rise to granulocytes (neutrophils, eosinophils and basophils), monocytes/macrophages, erythrocytes and megakaryocytes while CLP give rise to B- lymphocytes and T-lymphocytes (Moss, 2016).

1.2.2 The lymphoid and myeloid lineages

Traditionally, mature blood cells with specific functions are categorised into two different development lineages; the lymphoid lineage (B, T and NK cells) and the myeloid lineage (granulocytes and monocytes). It is believed that these two classes undergo separate differentiation pathways (Iwasaki & Akashi, 2007; Traver & Akashi, 2004). Dendritic cells have a specific development program that is usually activated from either lymphoid or myeloid pathways (Manz et al., 2001). The earliest lineage-decision that a developing haematopoietic stem cell or multipotent progenitor population must make is whether to develop into a lymphoid or myeloid lineage, and once done, that decision is irreversible (Kondo et al., 2003). The common lymphoid progenitor (CLP) is the first known lymphoid-restricted cell (Kondo et al., 1997), while the common myeloid progenitor (CMP) is the first known myeloid-restricted cell in the adult mouse bone marrow (Iwasaki & Akashi, 2007). These two progenitor populations represent the early branch points between myeloid and lymphoid lineages and interestingly, they have been shown to demonstrate a high level of lineage fidelity (restriction) in both *in vitro* and *in vivo* haematopoietic development studies (Akashi et al., 2000).

During commitment to either the lymphoid or myeloid pathways, progenitor cells undergo proliferation and sequential differentiation, which are accompanied by a decrease of regeneration capability to produce specific mature cells (Bellantuono, 2004). Each successive stage of development permanently loses the potential to become other specific cell types (Stuart, 2000).

1.2.3 The myeloid cells differentiation

Activation of specific transcription factors plays key role at the separate differentiation branches in determining lineage commitment. A number of genes that prevent normal myeloid cells development but have no effect on lymphocytes development have been identified (Metcalf, 1988). These genes encode transcription factors which regulate the expression of many myeloid genes, such as those encoding receptors for macrophage-colony stimulating factor (M-CSF), granulocyte-colony stimulating factor (G-CSF) and granulocyte/macrophage-colony stimulating factor (GM-CSF) as well as those encoding granule components, such as lactoferrin and neutrophil gelatinase (Rosenbauer & Tenen, 2007). The proliferation and differentiation of precursor cells committed to neutrophilic-granulocytic lineage is regulated by colony stimulating factors (Shimoda et al., 1997).

The gene encoding for Ets (E26 transformation-specific) family transcription factor PU-1, a product of oncogene SPI1 has been identified as the gene essential for the development of cells of the myeloid lineage (McKercher et al., 1996). Hence, formation of the early myeloid progenitors depends essentially on PU1. The PU.1 protein is exclusively expressed in haematopoietic cells and it has been linked to regulation of many genes of the myeloid lineage, such as c-FMS (CSF-1R) which encodes the M-CSF receptor. It is expressed at high levels in mature myeloid cells and contrastingly, its expression is down-regulated during early erythroid and T-cell differentiation in a step-wise manner, thereby contributing to myeloid specification (Dash & Gilliland, 2001; McKercher et al., 1996). Deletion of SPI1 in mice leads to defective foetal and/or new-born haematopoiesis, including complete absence of macrophages and B-cells (Metcalf, 1988; McKercher et al., 1996). Soluble cytokines, such as G-CSF and M-CSF secreted by stromal cells also play important roles at later stages of myeloid cell maturation to maintain myelopoiesis (Barreda, et al., 2004).

Retinoic acid receptors (RARs) are another class of transcription factors essential for normal maturation of neutrophils. Retinoid X receptors (RXRs) form heterodimers with RARs. Retinoic acid binds to RAR-RXR dimers when it enters a cell. This allows the dimer to enter the nucleus and bind to specific sites within the promoter sequences on specific regions of DNA, and enable RAR to regulate gene expression (Lutz et al., 2002).

1.2.4 Regulation of myeloid cells differentiation

All myeloid cells have a relatively short lifespan and thus must be replaced with newly produced ones, from the differentiation of haematopoietic stem cells present in the bone marrow. Their normal number in the bloodstream is maintained by the balance between newly-produced cells recruitment into tissues or cell death by apoptosis, the latter via a well-regulated process whose disruption could result in one of the various types of leukaemia. A key regulator of haematopoietic cell numbers is growth factor removal, levels of which are tightly regulated *in vivo*, to ensure normal levels of erythrocytes and leukocytes. Several experiments have indicated that haematopoietic cells in culture undergo apoptosis when they are deprived of a specific growth factors essential for their survival (Kennedy & Deleo, 2009).

1.2.5 Defects in myeloid cell differentiation: leukaemia

Leukaemia arises because of excessive production of many types of mature or immature haematopoietic cells in the bone marrow and blood, due to defects in the processes that regulate their normal proliferation and differentiation. These defects

are believed to be the result of disruption in individual genes associated with the regulatory processes of growth and differentiation, first identified when the physical appearance of chromosomes (Karyotype) from cells of a patient with chronic myeloid leukaemia was compared with that of normal leukocytes. The leukemic cells appeared to have a deletion in chromosomes 21 and 22 (named after the city of their discovery as Philadelphia chromosome). Since then, many chromosomal deletions and translocations in many forms of leukaemia have been identified using cloning techniques and fluorescence *in situ* hybridization (FISH). These have also enabled researchers to identify the specific gene product affected by the disruptions (Iwasaki & Akashi, 2007; Dash & Gilliland, 2001).

Myeloid leukaemias are classified as acute (AML) or chronic (CML) according to the duration of clinical symptoms, maturity of the affected cells and total leukocyte count (Kawamoto & Minato, 2004). The characteristic features of acute leukaemia are short duration symptoms, presence of several immature cells in the bone marrow and blood and high total leukocytes count. Chronic leukaemia is characterised by symptoms of long duration, many mature cells in the bone marrow and peripheral blood, and a low to highly raised total leukocytes count. Leukaemia can also be classified based on the morphology of predominant blood cells as myelocytic, monocytic and lymphocytic leukaemia (Lutz et al., 2002).

Certain haematopoietic diseases are also associated with defective apoptosis. For example, myelodysplastic syndrome (MDS) is characterised by decreased number of both progenitor and mature cells in the early stages, due to increased apoptosis in progenitor cells. However, elevated numbers of immature haematopoietic cells is later observed, which perhaps is due to apoptosis resistance. Myelodysplastic syndrome usually progresses to become acute myeloid leukaemia (Kawamoto & Minato, 2004).

1.3 The production and maturation of neutrophils: granulopoiesis

Neutrophils and other leukocytes of the immune system are produced from pluripotent stem cells present in the bone marrow during haematopoiesis. About 60% of all granulocyte precursors are committed to the production of neutrophils (Kennedy & Deleo, 2009). Granulocyte differentiation and maturation continues through series of steps that culminate in the formation of granules and exit of mature neutrophils from the bone marrow into the peripheral blood (Figure 1.2). This process of granulocyte formation takes ~6.5 days and is known as granulopoiesis (Bainton et al., 1971).

Following granulopoiesis, terminally-differentiated, mature neutrophils are then released into the bloodstream to circulate for ~10-12 h before migrating into tissues where they function for 1-2 days, then die by apoptosis and are cleared from the tissue by macrophages or other phagocytic cells (Savill et al., 1989; Fliedner et al., 1964). Infection may, however, prolong their lifespan to several days in an infected tissue (Edwards, 1994). Neutrophils constitute about 40-65% of total leukocytes in the circulation, and are found at concentrations of $3\text{-}5\times10^{6}$/mL blood (Pillay et al., 2010). Mature neutrophils also exist in the bone marrow, liver, lung and spleen as reservoirs (marginated pools), for rapid deployment during infection or inflammation (Summers et al., 2010). The daily turnover of neutrophils is approximately 5×10^{10} cells per day in a normal healthy adult human but this number may increase during infection to 5- to 10-folds (Lieber et al., 2004). Granulocytes are phenotypically distinct from lymphocytes and other circulating leukocytes based on multiple features, such as the granulated cytoplasm, multilobed nucleus and specific cell surface markers (Futosi et al., 2013).

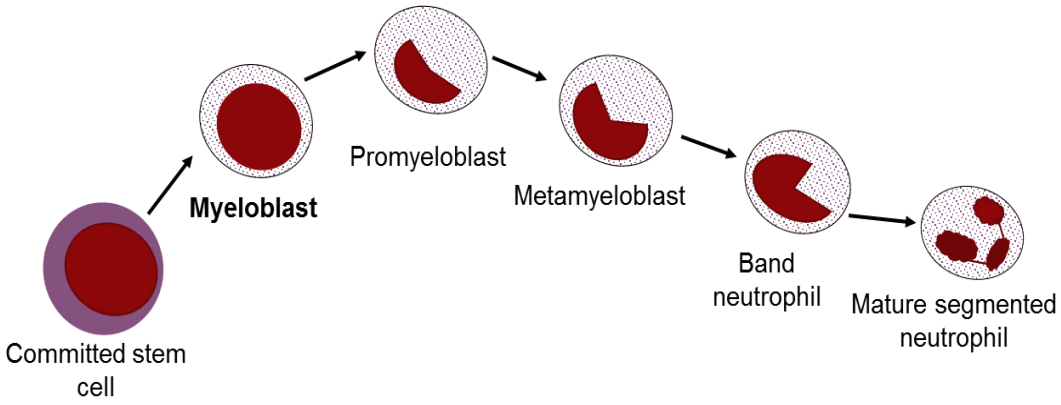


Figure 1.2 The stages of granulopoiesis. The early precursor myeloblast undergoes developmental and maturation stages to become a segmented mature neutrophil. During the granulopoiesis steps, neutrophils change their nuclear shape, undergo nuclear condensation, acquire granules in their cytoplasm and finally develop a segmented multi-lobular nucleus.

1.3.1 Neutrophil granules

Neutrophil granules are classified into three different types based on their protein contents and regulation of mobilization: primary granules (azurophilic or peroxidase-positive) contain myeloperoxidase (MPO), azurocidin and defensin; secondary granules (specific or peroxidase-negative) contain lactoferrin, gelatinase, ficolin 1 and cysteine-rich secretory protein 3 (CRSP3); tertiary granules (gelatinase) contain gelatinase B or matrix metalloproteinase 9 (MMP9) (Nauseef, 2007; Borregaard & Cowland, 1997). The biosynthesis of primary granules decreases during granulocyte maturation, whereas formation of secondary granules predominate as neutrophils mature (Kennedy & Deleo, 2009). These granules have different functions relative to their various protein and enzyme contents. For example, primary granules contain many cytotoxic proteins and peptides for killing and digestion of phagocytosed microbes, secondary granules supply proteins, such as receptors and oxidase components to the membrane and tertiary granules supply proteases for basement membrane digestion to allow for neutrophil extravasation and transmigration (Segal, 2005).

In addition, mature neutrophils possess secretory vesicles containing some proteins and receptors required for adhesion, transendothelial migration and chemotaxis. When transported to the plasma membrane, these vesicles can either release their proteins extracellularly or their membranes can fuse with the plasma membrane so that their proteins are expressed on the cell surface, for example, β2-integrin for rolling and adhesion (Borregaard, 2010; Borregaard et al., 1990).

1.3.2 Neutrophil membrane receptors

One of the remarkable features of neutrophils is the possession of different types of receptors on their plasma membranes which enable them to recognise and bind pathogens and other opsonised or un-opsonised particles. Neutrophil receptors can be divided into at least three groups (Hallett & Lloyds, 1995): (a) G-protein-coupled transmembrane receptors (GPCRs), including those for formylated peptides (FPRs) (Migeott et al., 2006), complement fragment 5a (C5a) (Chenoweth et al., 1978), platelet activating factor (PAF) (Lukashova et al., 2017) and interleukin-8 (IL-8) (Holmes et al., 1991), (b) single-transmembrane receptors activated through crosslinking or immobilisation, such as adhesion receptors (e.g. selectins and integrins) (Albelda & Buck, 1990) and immunoglobulin receptors (FcγRs) (Bruhns, 2012) and (c) single-transmembrane receptors, such as receptors for growth-regulating cytokines (e.g. GM-CSF, TNF-α) (Rose-John & Heinrich, 1994) and Toll-like receptors (TLRs) (Parker et al., 2005). These membrane receptors have different structures and mediate diverse functions differently. For example, activation of any of these receptors prime neutrophil NADPH oxidase for subsequent activation. However, GPCRs and crosslinking receptors (group a & b) prime the oxidase at lower concentrations and also activate it at higher concentrations, whereas the growth-regulating cytokine receptors (group c) prime but do not activate the oxidase, hence called ‘dedicated primers’ (Hallett & Lloyds, 1995).

1.4 Mobilisation and recruitment of neutrophils

Mobilisation of circulating neutrophils to the site of infection or inflammation constitutes a major event in the neutrophil functional responses and occurs very rapidly to attack and eliminate the invasive pathogen or mediate the process of

inflammation. Neutrophils are recruited through a series of controlled, exocytic events that change their functional status, converting them from ‘resting’ circulating cells to ‘active’ killing cells at the infection or inflammatory locus (Lee et al., 2003). Neutrophils reversibly move from the free circulation into the marginated pools, in which neutrophils are stored in capillaries of certain tissues for rapid mobilisation upon infection or cellular stress, and are much greater in number than the free-circulating neutrophils at any given time (Summers et al., 2010).

Neutrophils recognise chemoattractants including cytokines and chemokines (e.g. fMLP, LPS, GM-CSF, IL-8, TNF-α, INF-γ,) produced by host cells during infection or inflammation as ‘signals’ of microbial invasion and/or tissue injury or inflammation using their cell surface receptors. These molecules are released by microbes, injured host cells, macrophage engulfed-particles or following complement activation and recruit neutrophils to the site of infection or inflammation (Wright et al., 2010). Most of these chemotactic factors can also ‘prime’ neutrophils for enhanced function (Kobayashi et al., 2005).

1.4.1 Neutrophil priming

Circulating, inactive neutrophils become primed when exposed to infection-associated molecules, such as bacterial molecules (e.g. LPS and fMLP), cytokines (e.g. GM-CSF), leukotrienes, and activated complement proteins. Primed neutrophils are considered ‘ready to go’ but awaiting subsequent stimulation to trigger the functional response (Hallett & Lloyds, 1995). Priming induces gross morphological and cellular changes in neutrophils, including enhanced motility, polarisation and increased lifespan (thought to be mainly via stabilisation of the anti-apoptotic protein, Mcl-1). The increased lifespan provides neutrophils with sufficient time to enable them

perform their functions in infection or inflammation (Edwards et al., 2004). Priming also enhances the expression of cell surface receptors and adhesion molecules, by translocating intracellular stores on granule membranes to the plasma membrane (Edwards, 1994). During neutrophil priming, specific granules, gelatinase-containing granules and secretory vesicles fuse to the plasma membrane, thereby expressing increased number and/or affinity of surface receptors, and release of proteins to prepare the cells for transmigration (DeLeo et al., 1998).

1.4.2 Neutrophil transmigration and chemotaxis

Neutrophils exit peripheral blood circulation through the endothelial cells of the blood vessels to enter the sites of infection by a process called 'extravasation' (Futosi et al., 2013). Selectins, a C-type lectin family of glycoprotein expressed on the surface of activated endothelial cells (E- and P-selectins), primed neutrophils (L-selectin) and platelets (P-selectin) initiate the extravasation process. E- and P-selectins on the endothelial cells become up-regulated and interact with L-selectin on the surface of neutrophils to facilitate rolling of neutrophils along the surface of the endothelium (Kennedy & Deleo, 2009). The neutrophils then roll slowly until they stop and become attached to the activated endothelial cells. This initial interaction is called 'tethering' (Borregaard, 2010).

Following rolling, firm adhesion of neutrophils to the endothelial cells is promoted by leukocyte adhesion molecules (e.g. CD11b/18, Mac-1, CD54, and LFA-1) (Borregaard, 2010). The inflamed tissue releases chemokines, such as IL-8 into the luminal surface of endothelial cells, whereas the rolling neutrophils express chemokine receptors that bind these chemokines to increase the expression of adhesion molecules (β_2-integrins) and L-selectin shedding. The adhesion molecules

then mediate a stronger adhesion of neutrophils to the endothelial cells by interacting with ICAM-1 and ICAM-2 on the endothelial cells (Diamond et al., 1990). Other molecules expressed by neutrophils, including Mac-1, LFA-1 and PECAM-1 open the tight junctions of the endothelium for subsequent transmigration (Phillipson et al., 2006; Muller et al., 1993). Neutrophils then transmigrate through the endothelial cell gaps by 'diapedesis', a process that changes the morphology of neutrophils during chemotaxis (Wright et al., 2010; Sengeløv et al., 1993). Transmigration is mediated in part, by CD31 (PECAM-1) (Muller et al., 1993), CD44 (Khan et al., 2004), CD47 (Cooper et al., 1995) and CD54 (Diamond et al., 1990).

Neutrophils can transmigrate paracellularly (between endothelial cells) or intracellularly (through an endothelial cell), but they usually transmigrate paracellularly as this method is faster and more efficient (Figure 1.3). By the time neutrophils reach the site of infection, pathogens may have been opsonised by immunoglobulins, complement fragments or acute-phase reactant proteins. The opsonised particles can then be detected and engulfed by the neutrophils through opsono-phagocytosis, for destruction and elimination (Borregaard, 2010).

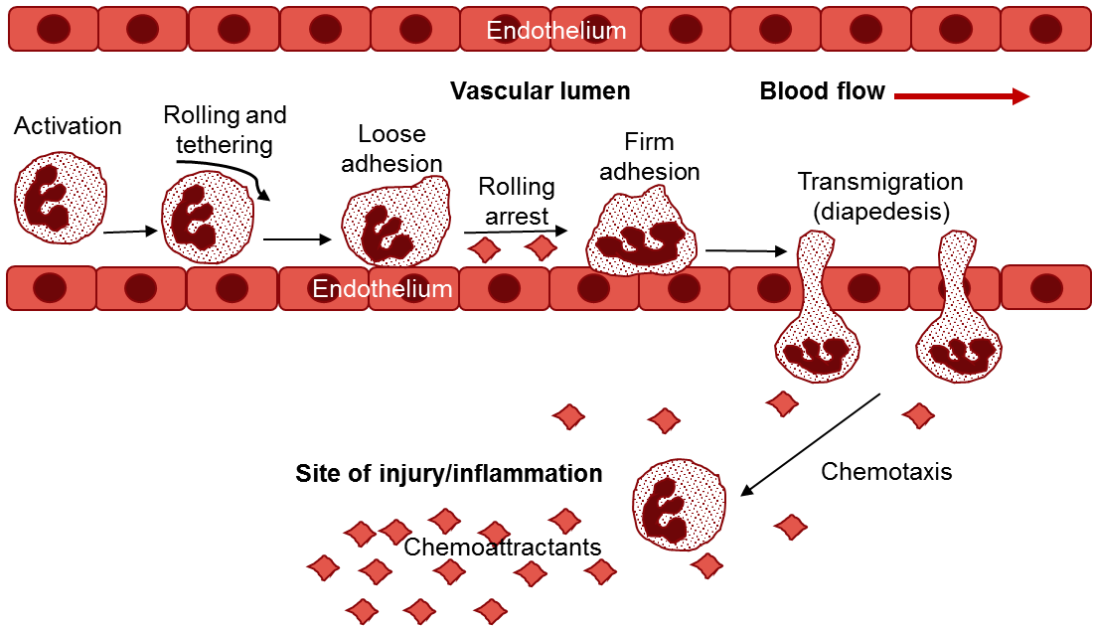


Figure 1.3 Transmigration and chemotaxis of neutrophils. Tethering interactions facilitate neutrophils rolling along the vascular endothelial wall mediated by selectins on endothelial cells and neutrophils. Host and/or pathogen derived chemotactic molecules, such as IL-8, TNF-α and LTB4 diffusing from the infection site into the bloodstream bind specific receptors on the neutrophil surface, thereby arresting the rolling and inducing firm adhesion to the endothelial cells, mediated by L-selectin and β2-integrin. Adhesion then leads to transmigration (para- and intra- cellularly) into the target tissue by diapedesis, mediated by LFA-1 and MAC-1 on the neutrophils as well as PECAM-1, ICAM-2 and CD99 on the endothelial cells. Once in the tissue, primed neutrophils migrate to the infection site by chemotaxis, where they recognise, bind and phagocytose the invading microbes (Rigby & DeLeo, 2012).

1.5 Microbicidal activity of neutrophils

Neutrophils are endowed with the capacity to ingest and subsequently kill pathogens through a series of well-coordinated and tightly-regulated processes of opsono-phagocytosis and oxidative burst, resulting from the enrichment of phagolysosomes with various cytotoxic molecules derived from cytoplasmic granules following degranulation (fusion of cytoplasmic granules with phagocytic vacuoles) (Lacy, 2006), production of reactive oxygen species (ROS) by the NADPH oxidase, hypohalous acids (e.g. HOCl) generated by the MPO-halide system, or generation of neutrophil extracellular traps (NETs), which entrap large pathogens that cannot be internalised by opsono-phagocytosis, such as fungi and protozoa and kill them extracellularly (Nakazawa et al., 2016).

1.5.1 Neutrophil phagocytosis

Phagocytosis is a phylogenetically conserved process by which leukocytes and other phagocytic cells engulf microbes and apoptotic or necrotic cell debris. Phagocytosis is critical to the microbicidal function of neutrophils, which along with macrophages comprise the professional phagocytes (Lee et al., 2003). Phagocytosis is also a fundamental aspect of tissue homeostasis and remodelling, where it is involved in the clearance of apoptotic bodies formed from the daily turnover of billions of cells (Flannagan et al., 2012). Neutrophils are the primary professional phagocytes recruited to the site of infection or inflammation and provide the first line of defence to the host against the invading microbes.

Neutrophils initially ingest particles into a plasma membrane-derived vacuole, the phagosome, following protrusion of pseudopodia which is then followed by a complex

process of maturation to acquire its degradative properties (Greenberg & Grinstein, 2002). The nascent phagosome itself is insufficient to mediate pathogen killing until the maturation process occurs, which transforms the phagosome into a potent, cytotoxic phagolysosome that is acidic, oxidising and hydrolase-rich (Figure 1.4). This transition involves influx and efflux of materials through the initial fusion of phagosomes to endosomes and subsequently to lysosomes, thereby acquiring protein recycling function of the endosome and degradative function of the lysosome (Pitt et al., 1992). In neutrophils, azurophilic and specific granules fuse with the phagosome to generate the phagolysosome (Edwards, 1994).

Special receptors on the plasma membrane of neutrophils must recognise and bind the target particles either directly via pattern-recognition receptors (PAMPs) or indirectly via opsonic receptors before uptake (Flannagan et al., 2012). Fcγ receptors on the neutrophil cell surface recognise and bind to IgG antibodies bound to bacteria (e.g. *Staphylococcus aureus* and *Escherichia coli)*. The principal Fcγ receptors in a resting neutrophil are the low affinity receptors, FcγRIIIb (CD16) and FcγRIIA (CD32), whilst in the primed neutrophil, the high affinity receptor FcγRI (CD64) may become functional (van de Winkel & Capel, 1993). Complement receptors, CR1 and CR3 (β2-integrin Mac-1 or CD11b/CD18) recognise complement fragments (C3a, C3bi and C5) (Neuman et al., 1990).

Activation of these receptors triggers the neutrophil cell membrane to extend pseudopodia. Following the formation of pseudopodia, neutrophils undergo morphological changes from spherical to an amoeboid shape that eventually surrounds and entraps the particle to form a phagocytic vacoule, phagosome. Next, the phagolysosome is formed by fusion of neutrophil granules to the vacoule (Borregaard, 2010). These granules contain various anti-microbial proteins and peptides, such as myeloperoxidase, elastase, proteinases and hydrolases, plus the

assembled and activated NADPH oxidase which generates ROS that together, kill and degrade the internalised microbe (Borregaard & Cowland, 1997). Neutrophils, like macrophages can ingest both opsonised and non-opsonised particles (Lee et al., 2003). Complement-opsonised particles are ingested by attachment and gentle sinking into the cell, in contrast to IgG-opsonised particles which are engulfed following extension of pseudopodia, initiated by ligation of Fcγ receptors (Kennedy & Deleo, 2009; Hed & Stendahl, 1982).

Neutrophils can also undergo 'frustrated' phagocytosis under certain pathological conditions. Frustrated phagocytosis occurs when the neutrophil attaches to a large, non-phagocytable surface, such as the cartilage-pannus junction in rheumatoid arthritis. This is triggered by the binding of IgG on the joint surface to the Fcγ receptors and subsequent activation of the neutrophils. However, the activated neutrophils are unable to internalise the surface as they do for small microbes, thereby creating a microenvironment of incomplete phagosome on the cartilage-pannus junction of the joint. This results in neutrophils degranulating directly onto the cartilage releasing proteases and ROS which degrade it and cause joint damage (Mohr & Menninger, 1980).

1.5.2 Neutrophil oxidative burst activity

The oxidative burst, otherwise called the respiratory burst, is a metabolic process that occurs during phagocytosis whereby phagocytes produce reactive oxygen intermediates (superoxide and hydrogen peroxide) that are either directly or indirectly toxic to the pathogens (Kim & Seoh, 2015). During phagocytosis, neutrophils increase their oxygen consumption due to the activities of the activated membrane-bound

NADPH oxidase, that results in the generation of reactive oxygen species (Kennedy & Deleo, 2009).

The multi-component NADPH oxidase complex is composed of at least 7 proteins that reside on the membrane and in the cytosol of the resting neutrophil. The enzyme complex becomes assembled and activated on the plasma membrane or within the phagosome. Upon activation, the cytosolic proteins translocate to the membrane where they associate with the membrane proteins to assemble the NADPH oxidase (Edwards, 1996). The assembled NADPH oxidase catalyses the formation of superoxide (O_2^-) by electron transfer from cytosolic NADPH onto extracellular (or intraphagosomal) molecular oxygen (O_2) (Dahlgren & Karlsson, 1999). The reaction catalysed by this enzyme complex is shown below:

$$NADPH + 2O_2 \longrightarrow NADP^+ + 2O^-_2$$

The transfer of electrons from NADPH to oxygen is a multi-step process involving sequential transportation of electrons along several components of the oxidase. FAD and two hemes are part of the redox centre of the cytochrome b_{558} component of the oxidase, but NADPH cannot directly bind this protein until the enzyme is completely assembled during activation, after which electron transfer occurs (Roos et al., 2003).

$$NADPH \longrightarrow FAD \longrightarrow 2\ Heme \longrightarrow 2O_2$$

Defects in bactericidal activity of neutrophils observed in chronic granulomatous disease (CGD) patients result from their inability to generate superoxide because one or more of the components of the oxidase are either absent or defective. However, superoxide has a low microbicidal potency, suggesting that it is perhaps, not the superoxide itself that kills microbes. Within the phagosome, the superoxide

spontaneously or enzymatically dismutates into hydrogen peroxide (H_2O_2), which then reacts with superoxide to generate hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($O^{\cdot}$), both highly reactive and toxic compounds (Kennedy & Deleo, 2009; Quinn et al., 2006).

$$^{\cdot}O_2^- + {}^{\cdot}O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$H_2O_2 + {}^{\cdot}O^-_2 \longrightarrow {}^{\cdot}OH + OH^- + {}^1O_2$$

Hydrogen peroxide may also, together with halide ions be used as a substrate by myeloperoxidase (MPO) released from azurophilic granules, to catalyse the formation of hypohalous acids, such as hypochlorous acid (HOCl), a very toxic compound for all microbes (Nauseef, 2007). NADPH oxidase and the MPO-halide systems are therefore, critical components of human neutrophil antimicrobial activity (Figure 1.4).

$$H_2O_2 + Cl \longrightarrow HOCl + H_2O$$

Hypochlorous acid is short-lived and can subsequently react with amines to form secondary chloramines, which are much more stable and as microbicidal as hypochlorous acid.

$$R\text{-}NH_2 + HOCl \longrightarrow R\text{-}HN\text{-}Cl + H_2O$$

Superoxide can also react with nitrous oxide (NO) generated by the inducible NO synthase to produce a very reactive nitrogen intermediate, peroxynitrite (Roos et al., 2003).

$$O_2^- + NO^{\cdot} + H^+ \longrightarrow OH^{\cdot} + NO_2$$

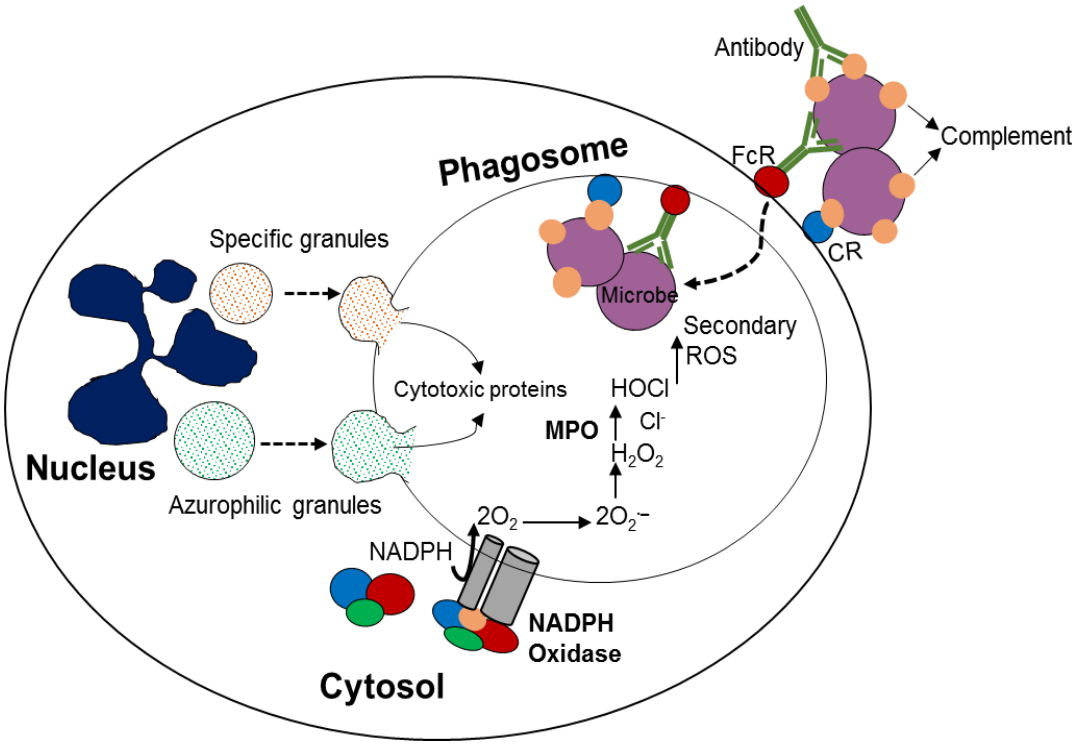


Figure 1.4 Neutrophil phagosome formation and generation of reactive oxidants. At the site of infection, primed neutrophils recognise and engulf the invading microbe leading to formation of the phagosome. Priming also facilitates assembly of the NADPH oxidase components at the membrane. This activates the oxidative burst and production of ROS (H_2O_2, O_2^-, $HO^{\cdot}$ and $^1O_2$) through oxidation of NADPH generated during the increased activity of the hexose monophosphate (HMP) shunt. Hypochlorous acid (HOCl) production occurs via myeloperoxidase (MPO), as well as generation of other secondary ROS which together kill the engulfed microbes within the phagosome (Quinn et al., 2006).

It has also been recently suggested that singlet electron may be converted into other compounds similar to ozone (O_3) in a reaction catalysed by neutrophils- or microbe-bound antibodies. In this way, neutrophils kill a large array of microbes very efficiently (Babior et al., 2003; Roos et al., 2003).

1.5.3 Neutrophil extracellular traps

Neutrophils may generate web-like structures, called neutrophil extracellular traps (NETs) which entrap and kill large extracellular pathogens, such as fungi and protozoa that cannot be engulfed by opsono-phagocytosis. During NETs formation, the activated neutrophils release their DNA and associated histone (chromatin) which then interact with granule enzymes to form a mesh-like structure. NETs are therefore, comprised of large strands of the de-condensed DNA and nuclear histones (chromatin), proteins from primary granules (e.g. elastase, myeloperoxidase and cathepsin G), secondary granules (e.g. lactoferrin) and tertiary granules (e.g. gelatinase) (Thieblemont et al., 2016; Nakazawa et al., 2016). However, several cytoplasmic proteins, granule membrane protein (CD63) and cytoplasmic markers, such as actin, tubulin and annexin 1 are absent from NETs (Brinkmann et al., 2004). NETs can be found *in vivo* in acute inflammations, such as dysentery and appendicitis (Brinkmann et al., 2004).

NETs trap microbes, prevent them from spreading and allow for elevated levels of antimicrobial molecules to degrade virulence factors and kill the microbes. NETs also serve as a source of autoantigens (Brinkmann et al., 2004). The efficiency of NETs as a new microbicidal mechanism has not yet been clearly established and their *in vivo* role remains controversial (Menegazzi et al., 2012). However, the release of NETs has been reported to represent a new form of neutrophil death called NETosis,

which is distinct from either apoptosis or necrosis, and which provides way for the neutrophils to continue killing pathogens beyond the end of their lifespan (Fuchs et al., 2007).

1.6 Control of neutrophil activity

Neutrophils are highly potent and non-specific killing cells. They may contribute to inflammatory damage in diseases such as rheumatoid arthritis, where inappropriately activated neutrophils accumulate in the joints and release reactive oxygen species and granule proteins into the extracellular milieu, causing swelling, localised tissue damage, and joint destruction (Cross et al., 2005). It is therefore, necessary to prevent inappropriate activation of neutrophils as this can lead to tissue damage or persistent inflammation. Neutrophils exist in either quiescent (inactive), primed or active states (Hallett & Lloyds, 1995). In the circulation, neutrophils exist as inactive cells undergoing constitutive apoptosis after 6-18 h (Edwards et al., 2004). Control of neutrophil function is largely achieved, by a two-step activation mechanism plus auto-regulatory processes, including spontaneous apoptosis. Neutrophils first become primed when exposed to infection-associated molecules and their full activation occurs once a pathogen is encountered. This two-step activation process normally prevents the risk of inappropriate activation.

Following clearance of infection or inflammation, the effete neutrophils undergo a safe and non-inflammatory process of cell death by apoptosis and the apoptotic bodies are subsequently cleared by macrophages (Lagasse & Weissman, 1994). The presence of cytotoxic enzymes and ROS within neutrophils, make it imperative that these cells are disposed of in a controlled manner, otherwise they would cause inflammation or localised tissue damage if these toxic molecules were released

following necrosis. For example in chronic obstructive pulmonary disease (COPD), infiltration of neutrophils followed by necrosis (thought to be induced by *Haemophilus influenza*) results in tissue damage (Naylor et al., 2007).

1.6.1 Apoptosis of human neutrophil

Kerr, Wyllie and Currie first coined the term 'apoptosis' in 1972, to describe an orderly and programmed cell death, which occurs through a series of coordinated events that results in the cellular contents being packaged into discrete vesicles, and disposed of safely by macrophages, upon recognition of apoptotic signals expressed on the surface of the dying cell (Kerr, 2002; Kerr et al., 1972). Well-regulated apoptosis is essential to fundamental life processes, such as morphogenesis during embryonic development, tissue-homeostasis for safe removal of damaged or excessive cells and for the resolution of inflammation (Jacobson et al., 1997). Dysregulated or delayed apoptosis, which will abnormally prolong the survival of an aberrant cell, can lead to persistent inflammation or to the development of cancers (Derouet et al., 2006; Igney & Krammer, 2002). Furthermore, increased resistance to apoptosis can make cancer cells difficult to kill using conventional cytotoxic or radiologic therapies (Vousden, 2001; Zangemeister-Wittke & Simon, 2001). On the other hand, excessive apoptosis leads to neurodegerative diseases, such as Alzheimer's and multiple sclerosis (Rohn, 2010). The morphological changes observed in all cells undergoing apoptosis are identical, leading to the conclusion that apoptosis was evolutionary 'conserved' and genetically 'programmed' in every cell (Afford & Randhawa, 2000).

Apoptosis enables an organism to remove old, damaged or unwanted cells through an orderly process of cellular death without inducing any undesirable inflammatory responses (Jacobson et al., 1997). The normal apoptotic process culminates in the activation of caspases, a family of cysteine proteases that act as effectors of the cell

death pathway and are responsible for the dismantling of the cell's components, which are subsequently packaged into smaller apoptotic bodies for clearance by phagocytes (Thomas et al., 2010). Upon their activation, caspases cleave specific proteins such as DNA-dependent protein kinase (DNA-PK), topoisomerases and some cell cycle regulators (Pucci et al., 2000). Caspases are also responsible for the morphological features observed in apoptotic cells, such as blebbed nuclear membrane structure, nuclear fragmentation, rounding and shrinking of the cell due to cytoplasmic and chromatin condensation. These features are exhibited when the cell reaches the final stages in the apoptotic pathway (Elmore, 2007; Vousden, 2001).

Apoptosis is distinct to necrosis, which is a disordered cell death, usually arising from cellular trauma, caused by external factors, such as toxins or physical injury, resulting in the cellular contents released into the external milieu, causing inflammation and local tissue damage by cellular enzymes, such as lysozymes and proteases (Naylor et al., 2007). As described previously (section 1.6), when human neutrophils complete their lifespan or their roles in infection and inflammation, they undergo constitutive apoptosis. Apoptotic neutrophils are non-functional and express distinctive surface molecules (e.g. phosphotidylserine) that allow for their safe recognition and elimination by macrophages and other phagocytic cells. This safe removal prevents tissue damage that would otherwise occur by release of neutrophils destructive products into the surrounding tissues if their apoptosis is delayed or should they die by necrosis.

1.6.2 Pathways of neutrophil apoptosis

Neutrophil apoptosis can be triggered by two major pathways: the *extrinsic* pathway or 'death receptor' pathway, activated by the engagement of death ligands with death receptors on plasma membrane (Akgul & Edwards, 2003), and the *intrinsic* pathway

or ‘stress pathway’, regulated by the mitochondria and the Bcl-2 protein family members (Akgul et al., 2001; Hengartner, 2000). In some cells, the extrinsic pathway links with the intrinsic pathway via the activated BH-3 only protein tBid (truncated Bid) which can engage Bcl-2 homology proteins, and possibly Bax (Adams & Cory, 2007).

1.6.2.1 The extrinsic apoptotic pathway

The *extrinsic* pathway of apoptosis is activated when death ligands bind to their cognate death receptors on the cell surface, thereby mobilising apoptotic proteins and caspase cascades inside the cell (Rossi & Gaidano, 2003). The caspase cascade is activated by activation of an initiator caspase 8, through cleavage of its pro-form into the active form, which then directly activates executioner caspases, such as caspase 3 and 7 and/or indirectly activates the *intrinsic* pathway through Bid cleavage, leading to formation of apoptosome, then triggering of apoptosis. This pathway can therefore, link to the *intrinsic* pathway via the cleaved Bid (tBid) (Fan, et al., 2005).

The *extrinsic* pathway is regulated by both pro-apoptotic factors, such as Fas ligand (FasL), TNF-α and TRAIL that trigger cell death, as well as anti-apoptotic factors, such as GM-CSF, G-CSF and IFNs that prevent cell death (Akgul & Edwards, 2003). FasR (Apo-1/CD95) and TNFR1 that bind Fas ligand and TNF-α, respectively are the two most characterized neutrophil death receptors and both activate the caspase cascade. Several adaptor proteins are recruited during engagement with the death receptors that facilitate interaction with other proteins (including pro-apoptotic proteins). These include TNF-associated death domain-containing proteins (TRADD) and Fas-associated death domain-containing proteins (FADD) (Ashkenazi & Dixit, 1998). For example, Fas clusters with FADD and pro-caspase-8 or DISC (death-inducing signalling complex) to activate pro-caspase-8 and initiate the caspase

cascade (Peter & Krammer, 2003), whereas TNFR1 clusters with TRADD, FADD and pro-caspase-8 and other adaptor proteins to trigger apoptosis (Cabal-Hierro & Lazo, 2012).

1.6.2.2 The intrinsic apoptotic pathway

The *intrinsic* pathway of neutrophil apoptosis is initiated by cellular stress at the inner mitochondrial membrane, such as growth factor removal, products of oncogenes and bacterial or viral infections, leading to activation of Bid. The active truncated Bid (tBid) induces the release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm. Cytochrome c then associates with, and mobilises apoptotic protease-activating factor-1 (Apaf-1) and ATP to activate caspase 9 leading to formation of the apoptosome, which in turn activates executioner caspases (e.g. caspase 3 and 7) and triggers apoptosis (Kennedy & Deleo, 2009; Green & Reed, 1998). Both the *extrinsic* and the *intrinsic* pathways therefore, finally activate executioner caspases 3 and 7 to induce neutrophil apoptosis, and Bid serves as a cross-link between the two pathways (Brenner & Mak, 2009).

The B-cell lymphocytic-leukaemia proto-oncogene-2 (Bcl-2) protein family are the main regulators of the *intrinsic* apoptotic pathway. These proteins are divided into two groups namely; *anti-apoptotic* proteins (e.g. Bcl-2, Bcl-X_L, Mcl-1, A1, Bcl-w, Bcl-B, and XIAP) and *pro-apoptotic* proteins (e.g. Bax, Bak, Bid, Bik and Bim), according to their functional contribution in the regulation of apoptosis. The pro-apoptotic members promote cell death by inducing the mitochondrial outer membrane permeability (MOMP) and release of cytochrome c, which then stimulates caspase activation via the apoptosome formation (Kuwana & Newmeyer, 2003).

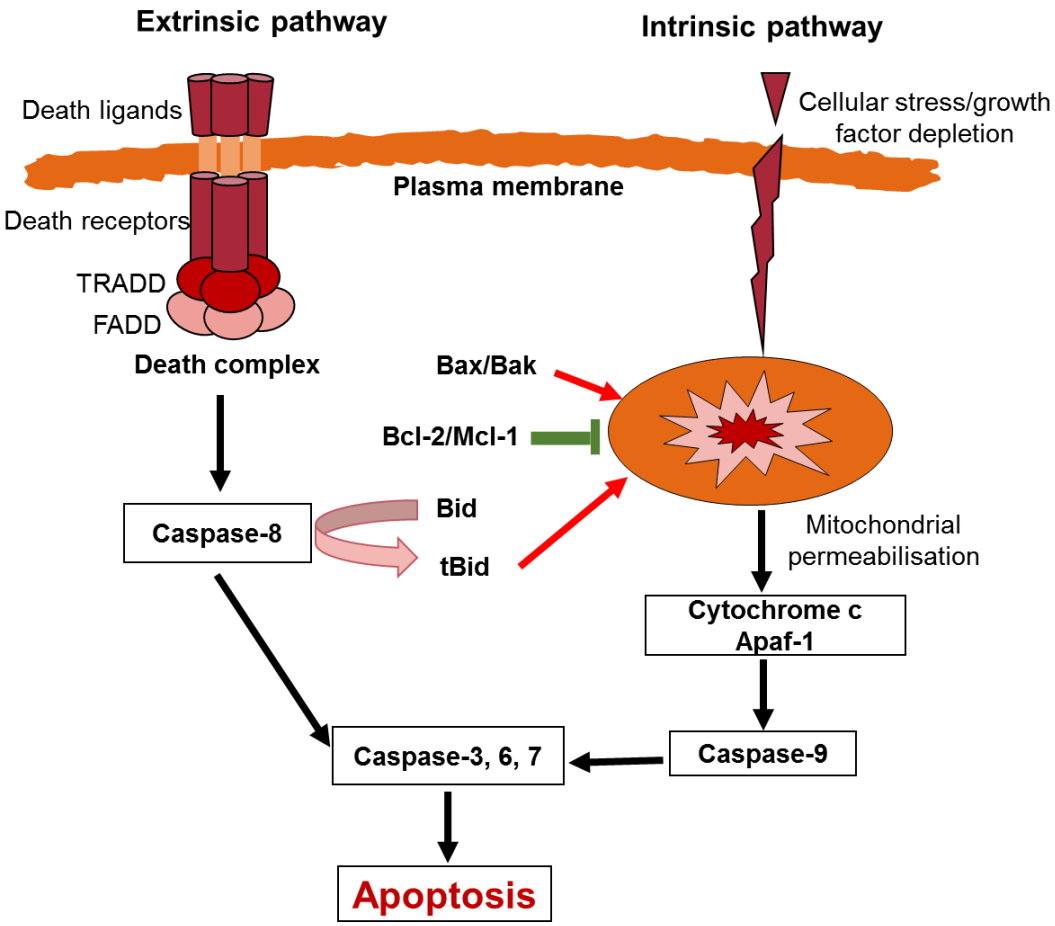


Figure 1.5 The pathways of apoptosis. The *extrinsic* pathway is initiated by binding of a death ligand to its receptor on the cell surface, resulting in recruitment of intracellular death and death engagement domains (DD) and subsequent activation of caspase-8 and caspase-3, leading to apoptosis. This pathway also engages the intrinsic pathway through cleavage of Bid to tBid by caspase-8. The *intrinsic* pathway is triggered by cellular stress at the mitochondrial membrane and is regulated by Bcl-2 protein family. Anti-apoptotic proteins, Bcl-2 and/or Mcl-1 sequester Bax/Bak oligomerisation and aBH3 (activator BH3) proteins. Upon stress, iBH3 (inactivator BH3) antagonises the release of aBH3 from Bcl-2/Mcl-1. This results in oligomerisation of Bax/Bak leading to permeabilisation of mitochondrial outer membrane (MOMP). Cytochrome c leaks out of the mitochondria and combines with Apaf-1 and ATP resulting in apoptosome formation, followed by activation of caspase-9, executioner caspase 3 and then triggering apoptosis (Adams & Cory, 2007).

The anti-apoptotic (pro-survival) Bcl-2 family proteins on the other hand, promote cell survival by keeping the mitochondrial membrane integrity intact, thereby protecting the release of cytochrome c from the intramitochondrial space (Brenner & Mak, 2009).

1.6.3 The Bcl-2 family proteins

The Bcl-2 protein family are the main regulators of the intrinsic apoptotic process and consists of about 20 members identified by having sequence homology to the eponymous Bcl-2 protein at one or more regions called BH (Bcl-2 homology) domains (Harada & Grant, 2003). The family is functionally divided into two groups namely; *anti-apoptotic* and *pro-apoptotic* members according to their functional contribution to the regulation of apoptosis. The balance between these two opposing groups of proteins determines the cell's fate towards survival or apoptosis (Brenner & Mak, 2009). When the activities of the *pro-apoptotic* proteins overcome those of the *anti-apoptotic* proteins, the cell progresses towards apoptosis (Mollet et al., 2013).

The two groups can be further sub-divided based on their BH domain structure. The anti-apoptotic members are multi-domain, containing three or four BH domains. For example, Bcl-2, Bcl-X_L and Bcl-W contain four BH domains (BH1-4) while Mcl-1 and A1(Bfl-1) contain three BH domains (BH1-3), lacking a well-defined BH4 (Akgul et al., 2000). The pro-apoptotic members are either multi-domain, containing three BH domains (e.g. Bax, Bak and Bok contain BH1-3 domains) or BH3-only (e.g. Bad, Bid, Bik, Bim, NOXA and PUMA contain only BH3 domain) (Akgul, 2009; Akgul et al., 2000). Furthermore, the BH3-only pro-apoptotic members have also been classified based on the nature of their interactions with other members of the Bcl-2 family into iBH3 (inactivator BH3-only) comprising Bad and NOXA proteins, and aBH3 (activator BH3-only) comprising Bid, Bim and PUMA proteins (Cairrão & Domingos, 2010; Thomas et al., 2010).

Table 1.1 The main Bcl-2 family members with their BH domain contents and apoptotic functions.

Anti-apoptotic (multi-domain)		**Pro-apoptotic**		
BH1-4	**BH1-3**	**Multi-domain (BH1-3)**	**BH3-only**	
			iBH3	**aBH3**
			Bad NOXA	Bid Bim PUMA

Mature blood neutrophils express a range of *pro-apoptotic* members of the Bcl-2 family proteins, which may partly explain their ability to undergo apoptosis spontaneously and rapidly, but Mcl-1 is the only *anti-apoptotic* Bcl-2 protein family member expressed in human neutrophils, that has been measured reliably at both the protein and mRNA levels, and whose levels correlate with the cell's survival (Edwards et al., 2004).

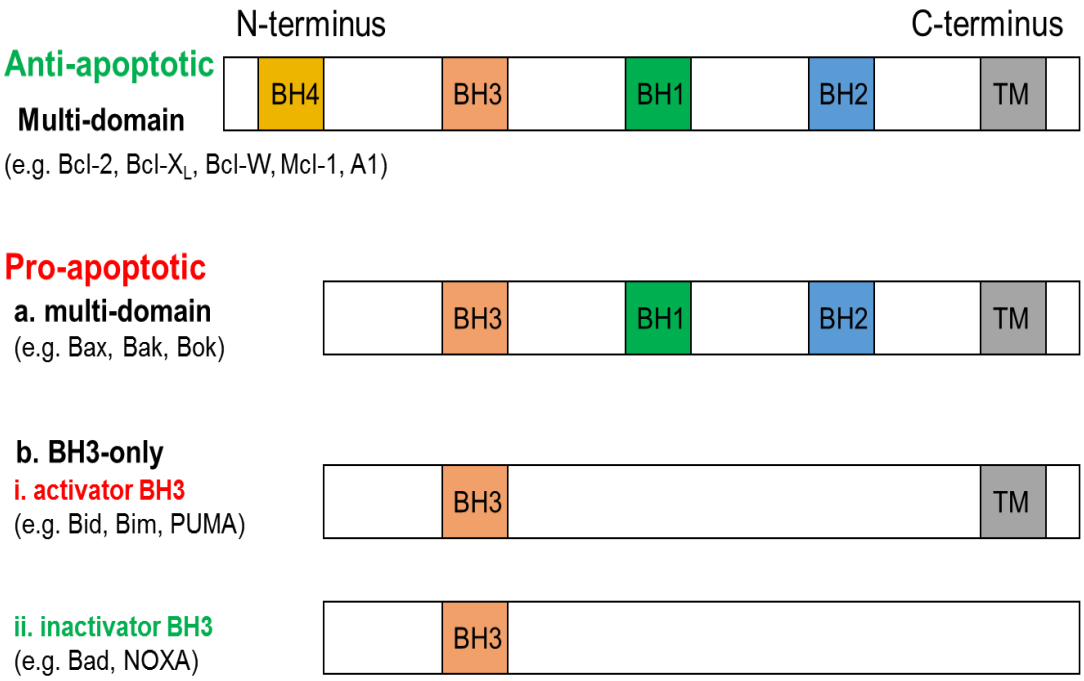


Figure 1.6 The schematic representation of BH-domain structure. All Bcl-2 protein family members contain a conserved BH (Bcl-2 homology) domain. Although these proteins share some degree of homology in their BH-domain structure, distinct structural differences exist between their BH domains that allow each member to carry out its specific function in the regulation of apoptosis. Most Bcl-2 members have a C-terminal TM (transmembrane) domain for anchoring into intracellular membrane, whilst the BH4 domain is restricted to some anti-apoptotic members only (Thomas et al., 2010) (Figure not drawn to scale).

1.6.3.1 Myeloid cell leukemia-1 (Mcl-1) protein

Myeloid cell leukemia-1 (Mcl-1) is an anti-apoptotic member of the Bcl-2 protein family, expressed in both normal and malignant myeloid cells (Mollet et al., 2013). It is a major regulator of apoptosis in myeloid cells and its cellular levels are closely correlated with apoptosis (Dzhagalo et al., 2008). Mcl-1 is highly regulated by transcriptional, translational and post-translational modifications, such as phosphorylation and ubiquitination (Edwards et al., 2004).

The MCL-1 gene was first identified as an early expressed gene during PMA-induced differentiation of the cell line, ML-1, and Mcl-1 protein was the second member of the Bcl-2 protein family to be discovered (Kozopas et al., 1993). Alignment algorithms have identified sequence similarities of Mcl-1 to other Bcl-2 proteins. The protein however, has some unusual properties not shared by other members of the Bcl-2 family. The large N-terminal domain of Mcl-1 determines many of its unique properties, and contains many motifs that affect its rate of turnover, localization and phosphorylation status. These post-translational modifications provide the protein with the ability to respond rapidly and reversibly to environmental signals, and switch the cell's fate from survival to apoptosis and *vice versa* (Lutz, 2000).

1.7 The cell cycle

The cell division cycle, simply known as the cell cycle, is a conserved process in multicellular organisms by which cells replicate themselves through a set of cyclic events, involving two major phases; S (DNA synthesis) and M (mitosis), leading to an accurate duplication of the cell without genetic abnormalities (MacLachlan et al., 1995). Replication of the genome is required for the transmission of genetic

information from one cell generation to the next and this occurs during the S phase of the cycle. The genome is then segregated into the two new daughter cells (nuclear division) during the M phase. In a normal cell cycle, M-phase does not occur until the S-phase is complete and is always preceded by the S-phase. DNA synthesis/replication and mitosis are therefore, the two essential processes triggered during the cell cycle (Pucci et al., 2000).

There are two preparatory gaps between S and M phases; Gap 1 or G1 prepares the cell for growth (growth phase 1) and separates the M phase from the S phase whilst Gap 2 or G2 prepares the cell for mitosis (growth phase 2) and occurs between the S and M phases. The cells grow and carryout all normal metabolic processes in growth phase 1. From G1, the cells that will undergo another division proceed to S phase, whereas those that cease division exit the cycle to enter a dormant state G0, which is a resting phase in which the cells stop growing and proliferating (Pucci et al., 2000; Yen et al., 1985). Overall, the events leading to cell division involve two major stages: (1) Interphase, which comprises the G1, S and G2 phases, and (2) Mitosis, which consists of prophase, metaphase, anaphase and telophase. Mitosis is followed by cytokinesis (cytoplasmic division) resulting in division of cell into two viable and identical daughter cells (Norbury & Nurse, 1992).

During haematopoiesis, cells undergo a limited number of divisions before they commit to differentiate towards a specific lineage. Once committed to a lineage, specific transcription factors are activated that result in changes (increases or decreases) in the expression of specific genes, which then change the molecular and phenotypic properties of the cell. These changes often limit proliferation, induce growth arrest and differentiation. The terminally-differentiated cells may then begin to die by apoptosis (Kim & Seoh, 2015).

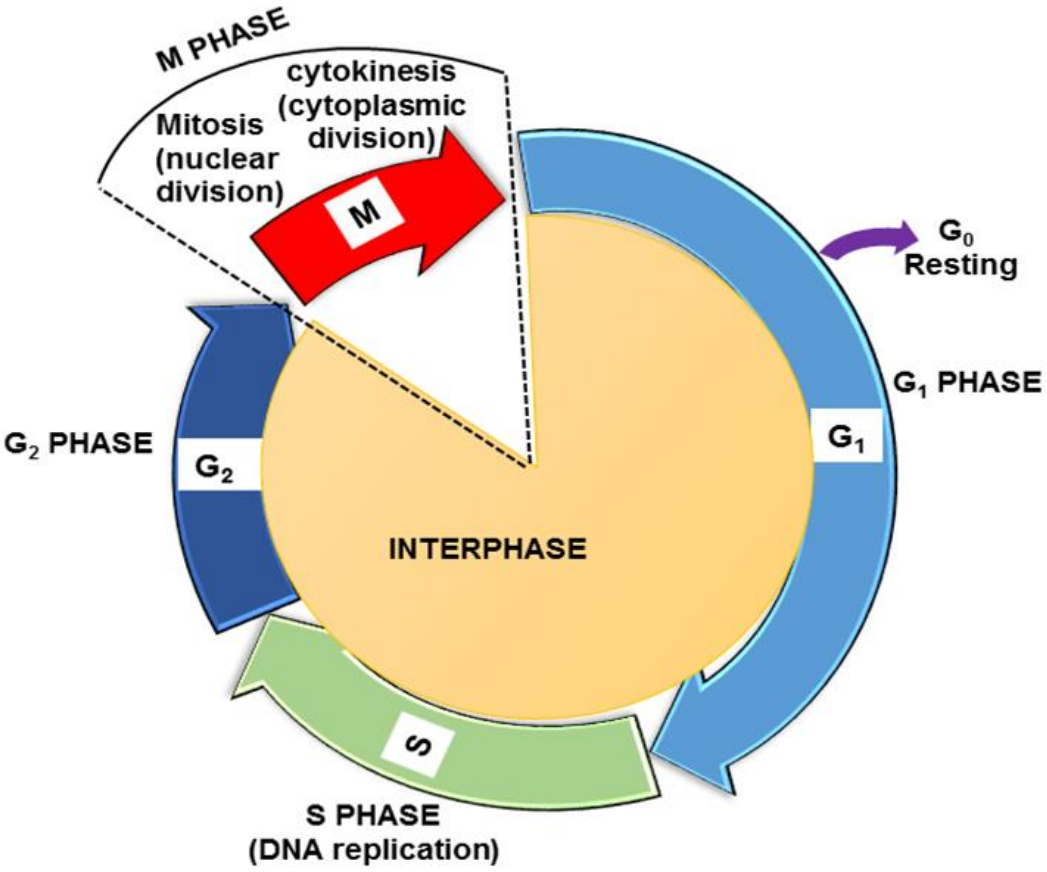


Figure 1.7 The phases of cell cycle progression. The eukaryotic cell division cycle comprises the three stages of interphase; G1, S and G2 and the Mitosis (nuclear division). G1 and G2 are phases of continuous cell growth while S is the phase of DNA replication. An inactive cell exits the cycle and enters the dormant state G0. Cells undergo two phase transitions; G1/S (to enter DNA synthesis), G2/M (to enter mitosis) during the cycle. This figure is however, not relevant to neutrophils as they are terminally-differentiated cells that do not undergo cell division cycle.

Circulating blood neutrophils are mature, terminally-differentiated and non-dividing cells. They have lost the ability to undergo the cell cycle or DNA synthesis and die constitutively by apoptosis (Edwards, 1994).

1.7.1 Regulation of cell cycle events

Cell cycle regulation is an example of homeostatic mechanisms that maintain proper development and cellular functions in multicellular organisms. The orderliness and timing of cell cycle events are checked at the G1/S and G2/M boundaries. These checkpoints involve control systems that ensure proliferation proceeds only in the presence of appropriate signals, such as growth factors as well as ascertain the fidelity of the genetic information passed from one generation to the next. For example, the checkpoints can be activated by DNA damage or by mis-aligned chromosomes at the mitotic spindle, in which cases cell growth is arrested to allow for repairs, because mutations or aberrations in the DNA will compromise the normal cell cycle control systems and perhaps lead to the development of cancers. Progression through the cell cycle resumes only after the damage is repaired, otherwise the cell is eliminated by apoptosis (Pucci et al., 2000).

The progression of the cell through the four phases of the cell cycle is regulated by activation and inactivation of members of the tyrosine/threonine family kinases called cyclin-dependent kinases (CDKs). CDK activity depends on the presence of activating sub-units of specific cyclins, whose abundance increases or decreases in the phases of the cell cycle where they are needed or not needed, respectively (Pucci et al., 2000) (Figure 1.8). CDKs can be divided into two groups based on their roles in cell cycle progression and genes transcription. The first group is involved in the regulation of cell cycle progression and includes CDK1, CDK2, CDK4 and CDK6.

CDK4 and CDK6 complex with cyclin D during the early G1 and their phosphorylation permits the transcription of genes required for entry into S phase. Once the cell is committed to enter into S phase, cyclin D-CDK activity is no longer required (Galaktionov et al., 1996). Activated CDK2 complexes with cyclin E or cyclin A during the G1-to-S transition and S phase progression, respectively (Ohtsubo et al., 1995). CDK1 complexes with either cyclin A or Cyclin B during the G2-to-M transition and M phase progression, respectively (see Figure 1.8) (Girard et al., 1991).

The second group of CDKs is involved in regulation of transcription, and includes CDK7/cyclin H and CDK9/cyclin T complexes. These promote the initiation and prolongation of RNA transcripts through phosphorylation of RNA polymerase II (Palancade & Bensaude, 2003). CDK activity is stimulated by dephosphorylating their conserved threonine and tyrosine residues via CDK-activating kinase (CAK), while the activity is down-regulated by phosphorylation of these residues via CDC25 phosphatases (Pucci et al., 2000; Galaktionov et al., 1996).

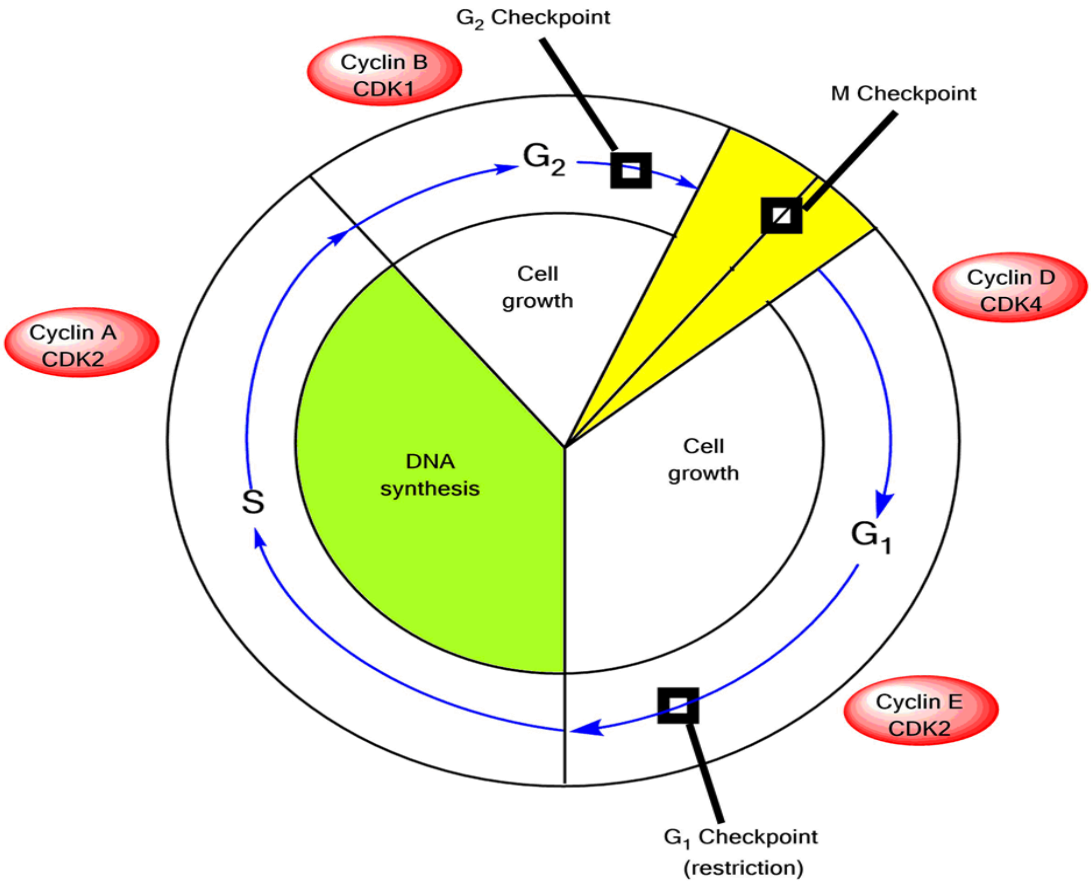


Figure 1.8 The cell cycle control checkpoints. The cell's progression through each phase of the cell cycle is regulated by CDKs and their associated cyclins (shown in red boxes) at different checkpoints within the cycle that determine whether or not the cell is prepared to enter into the next phase (Norbury & Nurse, 1992). This figure is also, not relevant to neutrophils as they are terminally-differentiated cells that do not undergo cell cycle.

1.8 The JAK/STAT signalling pathway

The Janus Associated Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway is a principal signalling mechanism for a wide array of cytokines and growth factors in mammals (Imada & Leonard, 2000). Janus associated kinases are a family of tyrosine kinases that play important roles in both innate and adaptive immunity, as well as in haematopoiesis. JAK family kinases consist of four members, JAK1, JAK2, JAK3 and TYK2 (tyrosine kinase 2). JAK1, JAK2 and TYK2 are expressed ubiquitously, but JAK3 is only expressed in haematopoietic cells (Clark et al., 2014), and has been described as the key regulator of Bcl-2 and Bax proteins (Edwards, 1994).

In addition, seven STAT proteins have been identified in mammalian cells, which are localised as clusters on different chromosomes; STAT1 and STAT4 on chromosome 1, STAT2 and STAT6 on chromosome 10, and STAT3, STAT5a and STAT5b on chromosome 11 (Rawlings, 2004). Nearly 40 different cytokine receptors signal through combination of these four JAKs and seven STATs, indicating common features across the JAK-STAT signalling cascade (Murray, 2007). Following binding of a ligand (cytokine or growth factor) to its cognate receptor, JAKs are activated, which in turn activates STAT proteins by tyrosine phosphorylation, leading to a rapid signalling from the plasma membrane to the nucleus where the activated transcription factors modulate expression of the target genes (Vijayakrishnan et al., 2010; Imada & Leonard, 2000). In the nucleus, the activated STAT proteins bind to specific promoter DNA sequences and initiate the transcription of genes that regulate essential processes in haematopoiesis and immune development, such as cell proliferation, differentiation and apoptosis (Figure 1.9) (Kisseleva et al., 2002).

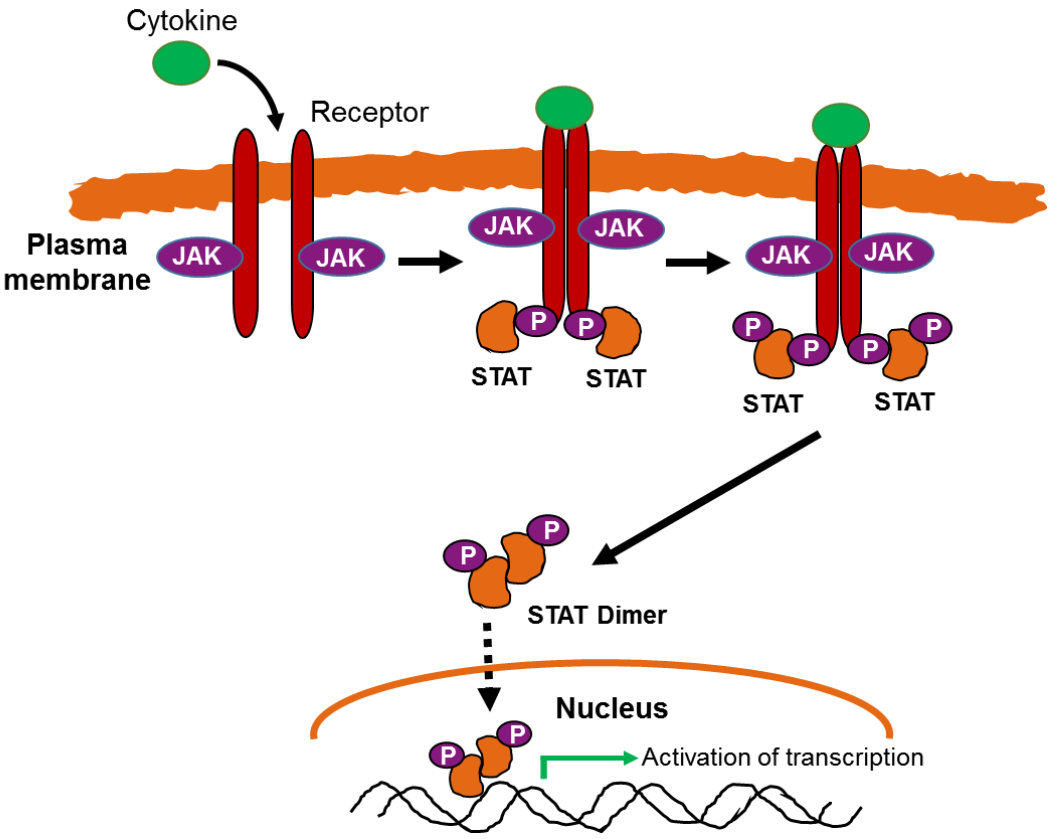


Figure 1.9 The JAK-STAT signalling pathway. In normal cells, the binding of ligand (cytokine or growth factor) to their receptors on the plasma membrane triggers receptor dimerisation and JAKs activation in the cytoplasm. The activated JAKs then phosphorylate the receptors to provide sites for recruitment and phosphorylation of STATs. The phosphorylated STATs then dimerise and translocate to the nucleus where they bind to the specific promoter sequences of the DNA to effect the transcription and expression of specific genes that regulate fundamental cellular processes (Rawlings, 2004).

Mutations that decrease or increase the activities of the JAK-STAT pathway negatively affect these processes, particularly mutations that constitutively activate or fail to regulate JAK-STAT signalling properly may result in inflammatory diseases and many forms of leukaemia (Rawlings, 2004).

1.9 Differentiation of leukaemia cell lines into granulocytes-like cells

The first demonstration of *in vitro* differentiation of leukaemia cell line into mature phenotype was in 1971, when Friend induced murine erythroid leukaemia (MEL) cells to differentiate into orthochromic erythroblasts *in vitro,* using dimethylsulfoxide (DMSO) (reviewed in Watanabe et al., 1988). Following this, several cell lines with the capacity to differentiate into mature neutrophil-like phenotypes along the granulocytic pathway have been established. These include: U-937 in 1974 (Sundström & Nilsson, 1976, Harris & Ralph, 1985), K-562 in 1975 (Andersson et al., 1979, Klein et al., 1976), HL-60 in 1977 (Collins, 1987), KG-1 in 1978 (Koeffler & Golde, 1978) and PLB-985 in 1985 (Tucker et al., 1987).

Moreover, a wide range of chemical agents have been used to induce the *in vitro* differentiation of leukaemia cell lines into mature phenotypes, including dimethylsulfoxide (DMSO) (Shin & Demura, 2012), dibutyrylcyclic AMP (dbcAMP) (Hazan-Eitan et al., 2006), granulocyte colony-stimulating factor (G-CSF) (Kim & Seoh, 2015), phorbol myristate acetate (PMA) (Perkins et al., 1991), 1,25-dihydroxy vitamin D3 (Perkins et al., 1991), interferon-γ (Hazan-Eitan et al., 2006), hexamethylenebiacetamide (HMBA) (Zhang et al, 2000), ester 12-0-tetradecanoylphorbol-13-acetate (TPA) (Delia et al., 1995), sodium pyruvate (Manz et al., 2001), retinoyl glucuronide (Zile et al., 1987), *N,N*-dimethylformamide (DMF)

(Katschinski et al., 1999) and *all trans* retinoic acid (ATRA) (Breitman et al., 1981; Breitman et al., 1980).

1.9.1 Human promyelocytic leukaemia PLB-985 cell line

The promyelocytic leukaemia cell line, PLB-985 is a sub-clone of the myeloid leukaemia cell line, HL-60. PLB-985 cell lines are less mature than the promyelocytes of the HL-60, thus they can be used to study earlier events involving commitments into granulocytes or monocyte/macrophage differentiation, that are not afforded by HL-60 (Tucker et al., 1987). These cells have been established from the peripheral blood of 38-year-old patient (woman) with a refractory myeloblastic leukaemia and are myelomonoblast-like, phenotypically(Pivot-Pajot et al., 2010, Pedruzzi et al., 2002, Tucker et al., 1987).

Promyelocytic leukaemia (PML) is a subtype of acute myeloid leukaemia (AML) characterised by cells that have a reciprocal translocation between chromosomes 15 and 17, which generates a fusion transcript joining the promyelocyte (PML) gene and retinoic acid receptor-α (RAR- α) gene (Rowley et al., 1977). The biologic and clinical features of the disease include leukopenia and life-threatening coagulopathy. The leukaemic promyelocytes, upon exposure to retinoic acid have the unique ability to undergo differentiation, and both differentiation and apoptosis when they are exposed to arsenic trioxide (Grignani et al., 1993).

PLB-985 is a bipotential cell line that can undergo both granulocyte and monocyte/macrophage differentiation in the presence of appropriate inducing agents. It is a more recently-established cell line that has been demonstrated to show greater levels of differentiation and is described as the current ‘best’ model of neutrophil differentiation (Hauert et al, 2002, Tucker et al., 1987). The PLB-985 cell line has

been widely used to measure expression of NADPH oxidase and production of reactive oxygen species (ROS) (Boulven et al., 2006), but other functional processes such as degranulation and phagocytosis are less well characterized, or restricted to one type of differentiating agent (Trayner et al., 1998).

Though varying degrees of PLB-985 differentiation have been reported, many features that are characteristics of mature neutrophils, such as polymorphic nucleus, are not always reported. Also, a major experimental limitation of these *in vitro* differentiation systems is that once differentiated, the neutrophil-like cells undergo apoptosis. Thus, new ways to delay apoptosis in differentiated PLB-985 cells would be of benefit as that would enhance the utility of the differentiation system, especially when trying to define the effects of knock-out or knock-in of genes on neutrophil function. In this project therefore, PLB-985 cells have been induced to differentiate *in vitro*, along the granulocytic pathway into neutrophil-like phenotypes and used as model to study neutrophil differentiation and functions. New experimental procedures that delay apoptosis of the differentiated cells have also been investigated.

1.10 Summary

Neutrophils are indispensable phagocytic cells of the immune system that circulate in the blood in a terminally-differentiated, inactive state undergoing constitutive apoptosis. They transform into active killing cells at the site of infection inflammation upon exposure to a variety of stimuli that trigger intracellular signal transduction pathways. Neutrophils also play pathological roles in the progression of autoimmune and inflammatory diseases, such as rheumatoid arthritis and systemic lupus erythematosus. A clear understanding of the molecular signalling processes of neutrophils in health and disease is necessary, but certain limitations, such as short

lifespan, lack of proliferation capacity and difficulty to transfect to express exogenous genes or proteins, have made the experimental manipulation of neutrophils *ex vivo* challenging, and hindered the biochemical studies of their signal activation mechanisms and genetic makeup.

Therefore, establishment of validated myeloid cell lines capable of differentiating into mature neutrophil-like phenotypes, as well as acquire the molecular and functional properties of mature neutrophils would be beneficial in addressing these limitations. In that regards, this research project investigated the potential of human PLB-985 cell line towards granulocyte differentiation and has characterised the molecular properties of the differentiated cells, as model of neutrophil differentiation and functions.

1.11 Research aim and objectives

The aims of this thesis are to develop a modified differentiation protocol and new optimisation procedures to obtain terminally-differentiated PLB-985 cells that resemble mature neutrophils morphology with prominent multi-lobed nucleus and granulated cytoplasm, as well as with appreciably extended lifespan. It also aims to study the molecular and functional properties of the neutrophil-like differentiated PLB-985 cells, in order to establish their usefulness as model of neutrophil differentiation and functions.

The main objectives of the study were:

1. To develop a modified differentiation protocol and new optimisation procedures to obtain differentiated PLB-985 cells, using a combination of

three differentiation- and maturation-inducing agents; ATRA, DMF and sodium pyruvate.

2. To induce PLB-985 cells to terminally differentiate into mature neutrophil-like granulocytes, with prominent multi-lobed nucleus, granulated cytoplasm and appreciably extended lifespan.
3. To characterise the molecular functional properties of the differentiated PLB-985 cells, in order to establish their usefulness as model of neutrophil fuctions, such as apoptosis, chemotaxis, phagocytosis, oxidative burst activity, expression of cell surface markers and activation of intracellular signalling pathways.
4. To determine the effects of small molecule JAK inhibitors, baricitinib and tofacitinib on the growth, differentiation and viability of the differentiated PLB-985 cells.

1.12 Hypothesis

The main hypothesis tested in this project is that “The human myeloid cell line PLB-985 can be induced to differentiate terminally into mature neutrophil-like phenotypes with an enhanced lifespan under appropriate conditions, and the differentiated PLB-985 cells can acquire most of the functional properties of mature blood neutrophils”.

Chapter 2: Materials and Methods

2.1 Materials

The promyelocytic leukaemia PLB-985 cell line was generously provided by Professor S.W. Edwards, University of Liverpool. RPMI 1640 (+10mM L-glutamine), RPMI 1640 + HEPES (+10mM L-glutamine), foetal calf serum (FCS), penicillin/streptomycin and Annexin V-FITC were obtained from Invitrogen (Paisley, UK), polymorphprep was from AxisShield (Oslo, Norway), rapid Romanowsky stains were from HD Supplies (Aylesbury, Bucks), *all trans* retinoic acid (ATRA) (R2625), ficoll paque, *N,N*-dimethyl formamide (DMF), sodium pyruvate, polyclonal rabbit anti-human Mcl-1 antibody, cycloheximide, tetramethylethylenediamine (TEMED), sheep HRP-linked anti-mouse IgG antibody and human pooled AB serum were from Sigma-Aldrich (Dorset, UK), anti-Actin monoclonal mouse antibody (Ab3280) was from Abcam (Cambridge, UK), anti-Bcl-2 antibody (#2876), anti- Bcl-X_L antibody (#2762), anti-Bax antibody (#2772), anti-Bak antibody (#3814), anti-biotin HRP-linked antibody and biotinylated molecular weight marker were from Cell Signalling (Hertfordshire, UK), Immobilin psq PVDF membrane, Immobilin western chemiluminescent HRP substrate and Guava viaCount reagents were from Millipore (Watford, UK), bis-acrylamide was from Geneflow (Hertfordshire, UK), BCA protein assay reagents were from Thermo Scientific (Southend-on-sea, UK), FITC-conjugated anti-human CD11b antibody (#130-091-240) was from Miltenyl Biotech, perCP-conjugated anti-human CD14 antibody (#FAB3832C) was from R&D Systems (Minneapolis, USA), monoclonal mouse anti-human Mcl-1 antibody and FITC-conjugated anti-human CD16 antibody (#555406) were from BD Bioscience (Oxford, England), FITC-conjugated isotype antibody control (#Sc-2855) was from Santa Cruz Biotechnology (Texas, USA), JAK inhibitotrs; baricitinib (#S2851) and tofacitinib (#S5001) were from

Selleckchem (Houston, USA), non-fat dry milk was from Home Bargains (Liverpool, UK), western blot equipment was from Bio-Rad (Hertfordshire, UK), all other reagents were from Sigma-Aldrich (Dorset, UK), all laboratory plastic ware were from Nunc (Denmark) and Greiner Bio-one (Germany).

2.2 Isolation of human blood neutrophils

Whole blood was collected from healthy donors by venupuncture, into heparinised tubes, under sterile conditions. Ethical approval was obtained for this study from the University of Liverpool Committee on Research Ethics (CORE). All participants gave written, informed consent. Polymorphonuclear leukocytes were separated by centrifugation using Ficoll-paque according to manufacturer's instructions. Cells were re-suspended in RPMI-1640 + HEPES (10mM L-glutamine) media and then contaminating erythrocytes removed by hypotonic lysis using ammonium chloride buffer (155 mM NH_4Cl, 13.4 mM $KHCO_3$, 96.7 µM EDTA, PH 7.6), in a 1:9 cell media to lysis solution ratio, for 3 min followed by centrifugation at 1,000 g for 5 min. Cells were counted using a Multisizer3 cell counter (Beckman Coulter) following a 1:1000 dilution with Isoton II and the suspension volume adjusted to give a final concentration of 5 $\times 10^6$ cells/mL using RPMI-1640 + HEPES (10mM L-glutamine) medium. Cell purity was assessed by cytospining (20 µL (1×10^5 cells) in 200 µL PBS (+EDTA) at 500 g for 5 min in a Shandon3 cytocentrifuge, and routinely found to be >95%, as determined by morphological examination under a light microscope, after rapid Romanowsky staining of cytospin slides. Cell Viability measured by Guava viaCount was also routinely >95%. Cells were usually incubated at 37^{o}C with gentle agitation, where incubations for ≥ 5 h, cells supplemented with 10% (v/v) human AB serum.

2.3 Culture and differentiation of PLB-985 cells

The human myeloid leukaemia PLB-985 cell line was cultured in RPMI-1640 (+10mM L-glutamine) medium (supplemented with 10% (v/v) foetal calf serum and 1% (v/v) penicillin/streptomycin) and incubated at 37^{o}C in a humidified atmosphere of 5% CO_2. Cell cultures were passaged every 2 - 3 days or as indicated in the text. For induction of differentiation into granulocytes, the RPMI-1640 (+10mM L-glutamine) medium was supplemented with 0.5% (v/v) foetal calf serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) sodium pyruvate, 0.5% (v/v) *N,N*-dimethylformamide (DMF) and 1µM *All-trans* retinoic acid (ATRA). Normally, the culture was passaged and re-suspended in fresh medium on days 2 and 4. The exponentially-growing PLB-985 cells were seeded at a starting density of 2×10^5/mL in separate culture flasks labelled dPLB-985 (differentiation-induced) and PLB-985 (non-induced) cells for a total of 6/7-day incubation period.

For use in experiments, cells were harvested and analysed each day or on selected days, depending on the experimental design. Cells were centrifuged at 1,000 g for 3 min and the supernatant discarded. Cells were re-suspended in fresh media, counted using a Multisizer3 cell counter (Beckman Coulter) and the suspension volume adjusted with media to give a final concentration of 1×10^6/mL. Cells were then plated under different experimental conditions as described in the text.

Table 2.1 Compositions of the culture media

Ingredients	Routine medium	Differentiation medium
RPMI 1640 + 10mM L-glutamine.	500 mL	500 mL
Fetal calf serum (FCS)	10% (v/v)	0.5% (v/v)
Penicillin/Streptomycin	1% (v/v)	1% (v/v)
Sodium pyruvate	-	1% (v/v)
N, N-dimethylformamide (DMF)	-	0.5% (v/v)
All-trans retinoic acid (ATRA)	-	1µM

2.4 Morphological analysis of PLB-985 cells differentiation

Following culture of PLB-985 cells in the presence and absence of differentiation-inducing agents, 20 µL of cells at a concentration of 1×10^6 /mL were diluted with 180 µL PBS (+EDTA), slides prepared in a Shandon3 cytospin and cytocentrifuged at 500 × g for 5 min to fix the cells on the slides. The slides were air-dried, stained with rapid Romanowsky stain and cell morphology analysed under a light microscope. Large rounded nucleus with well-defined nucleoli and agranular cytoplasm were observed in non-differentiated PLB-985 cells while the differentiated cells appeared with segmented, multi-lobed nucleus and granulated cytoplasm.

2.5 Morphological analysis of neutrophil and dPLB-985 cells apoptosis

Following appropriate incubations, cytospin slides were prepared as described (section 2.4) and stained with rapid Romanowsky stain, before they were examined microscopically for neutrophils or differentiated PLB-985 cells morphology. Viable neutrophils and dPLB-985 cells exhibited indented, convoluted and segmented nuclei, decreased nucleoli and granulated cytoplasm, whilst both apoptotic neutrophils and dPLB-985 cells appeared smaller with dense cytoplasm, condensed chromatin and round, compact nuclei.

2.6 Priming of isolated neutrophils and dPLB-985 cells

Neutrophils at a final concentration of 5 x 10^6 /mL or PLB-985 cells at 1×10^6 /mL were primed by incubation with either G-CSF (10 ng/mL) or GM-CSF (5 ng/mL) at 37ºC for 30 min with gentle agitation. This was followed by subsequent incubations according to various experimental designs.

2.7 Preparation of whole-cell lysates

Following culture and incubation of cells with the various treatments described in the text, for the indicated length of time, aliquots of 5×10^6 cells (neutrophils) or 1×10^6 cells (PLB-985) were pelleted by centrifugation at 1,000 g for 3 min, the supernatant discarded and cells re-suspended in 1mL of phosphate-buffered saline (PBS) to remove all traces of media. Cells were pelleted once again at 1,000 g for 3 min and supernatant discarded.

For PLB-985, cells were then re-suspended in 100 µL of boiling Laemmli lysis buffer (minus dithiothreitol and bromophenol blue) for 4-5 min with occasional vortexing. The cell lysates were then used for analysis or stored at -20^{o}C. For neutrophils, the PBS washing step was omitted and cells were re-suspended in 100 µL of boiling normal Laemmli lysis buffer (plus DTT and bromophenol blue), for 4-5 min with occasional vortexing. The cell lysates were then used for analysis or stored at -20^{o}C.

2.8 Bichinconinic acid (BCA) assay

For PLB-985 whole-cell lysates (protein extracts), the bichinconinic acid assay was performed as per manufacturer's instructions, to ensure all samples had equal total protein concentrations. The protein concentrations were measured using a spectrophotometer at an absorbance of 562 nm. Following determination of protein levels, samples were diluted with Laemmli buffer, 10% (v/v) dithiothreitol (DTT) and 1% bromophenol blue (0.01% w/v) added to make the final volumes of the samples. Prepared samples were used directly for analysis or stored at -20^{o}C.

2.9 Electrophoresis and Western blotting

Cell lysates were separated by SDS-PAGE using 1.5 mm thick resolving gels (12% (v/v) polyacrylamide, 370 mM Tris-HCl, pH 8.8, 1% (w/v) SDS, 1% (w/v) APS, 0.1% (v/v) TEMED). The APS and TEMED were added last to prevent polymerisation before casting. Following casting of resolving gels, water-saturated t-amyl alcohol or isopropanol was overlaid to ensure a levelled top surface of the gels. After polymerisation, the overlaid alcohol was discarded and gel's surface washed with

deionised water. Stacking gels (4.5% (v/v) polyacrylamide, 122 mM Tris-HCl, pH 6.7, 1% (w/v) SDS, 1% (w/v) APS, 0.1% (v/v) TEMED) were then cast onto the surface of resolving gels and a 10 or 15 wells casting comb inserted. After full polymerisation of the gels, samples of protein extracts were boiled for 5 min, centrifuged briefly and 15-20 µL were loaded per well. 10 µL prestained marker was loaded in lane 1 and 5 µL of biotinylated marker was loaded in the last lane. Gels were electrophoresed for 45-60 min at 200V using SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS).

Following separation, the stacking gel was removed and the resolving gel was allowed to equilibrate for 5 min in a transfer buffer (20% (v/v) methanol, 95mM glycine, 12.5 mM Tris). Polyvinylidene difluoride (PVDF) membrane was immersed in methanol for 30 sec and washed in transfer buffer. The gel and PVDF membrane were then assembled into a sandwich cassette for transfer. Proteins were transferred to PVDF membrane by electrophoresis at 100V for 1 h.

After protein transfer, membranes were briefly stained with ponceau S stain (0.1% (w/v) ponceau S in 5% (v/v) acetic acid) to confirm successful transfer, which was then removed with wash buffer (TBS (10 mM Tris, 150 mM NaCl, pH 8.0), 0.1% (v/v) Tween 20). Membranes were subsequently incubated for 1 h in a blocking buffer (TBS (10 mM Tris, 150 mM NaCl, pH 8.0), 0.1% (v/v) Tween 20) containing 5% (w/v) non-fat dry milk) at room temperature with gentle agitation to block non-specific antibody binding. Membranes were then washed 2×30 sec in wash buffer to remove the blocking buffer. Membranes were then incubated in antibody buffer (containing either 5% (w/v) non-fat dry milk or 5% (w/v) BSA in wash buffer), with specific primary antibody at an appropriate concentration (according to the manufacturer's instructions), overnight at 4^{o}C with gentle agitation. Membranes were washed thoroughly (3×5 min) with wash buffer to remove primary antibody, and then

incubated with specific horseradish-peroxidase (HRP)-conjugated secondary antibody in antibody buffer for 1 h, at room temperature on an orbital shaker. The membranes were once again washed with wash buffer (3×5 min) to remove the secondary antibody. Membranes were then treated with excess ECL detection reagents, incubated at room temperature for 5 min, dried, prepared in cassettes and exposed to photosensitive hyperfilm for 1-20 min depending on the signal intensity. The hyperfilms were then developed to detect the protein bands.

Following development of the blot, membranes were stripped for 15-30 min in a stripping buffer (50 mM Glycine and 150 nM NaCl, pH 2.5) and 0.1% (v/v) tween 20) before use for re-probing. The membranes were blocked in 5% non-fat dry milk (in wash buffer) at room temperature for 1 h, and then re-probed for other proteins of interest or for Actin to confirm equal protein loading in each lane. Table 2.2 summarises the antibodies used in western blotting analysis.

2.10 Densitometry

The intensity of the signals formed by the protein bands on x-ray hyperfilm following treatment with ECL reagents was quantified using a Scanner and AQM Advance 6 software by kinetic imaging, and the results were displayed graphically.

Table 2.2 Antibodies used for western blotting.

Protein of interest	Antibody buffer	Primary antibody (Dilution)	Secondary antibody (Dilution)	Molecular weight (KDa)
Mcl-1	Non-fat dry milk	Rabbit anti-human Mcl-1 (1:10,000)	HRP-linked donkey anti-rabbit IgG (1:10,000))	40
Mcl-1	BSA	Mouse anti-human Mcl-1 (1:1,000)	HRP-linked sheep anti-mouse IgG (1:10,000)	40
Bcl-2	BSA	Rabbit anti-human Bcl-2 (1:1,000)	HRP-linked donkey anti-rabbit IgG (1:10,000)	26
Bcl-X_L	BSA	Rabbit anti-human Bcl-X_L (1:1,000)	HRP-linked donkey anti-rabbit IgG (1:10,000)	30
Bak	Non-fat dry milk	Rabbit anti-human Bak (1:1,000)	HRP-linked donkey anti-rabbit IgG (1:10,000)	25
Bax	BSA	Rabbit anti-human Bak (1:1,000)	HRP-linked donkey anti-rabbit IgG (1:10,000)	20
Phospho-STAT3	BSA	Rabbit anti-human Phospho-STAT3 (1:1,000)	HRP-linked donkey anti-rabbit IgG (1:10,000)	79, 86
STAT3	BSA	Rabbit anti-human STAT3 (1:1,000)	HRP-linked donkey anti-rabbit IgG (1:10,000)	79, 86
β-Actin	Non-fat dry milk	Mouse anti-actin (1:20,000)	Sheep anti-mouse HRP-linked (1:10,000)	42

2.11 PI-labelling and complement-mediated opsonisation of S. aureus

To assay phagocytosis of *S aureus* by neutrophils and dPLB-985 cells, 1×10^{10}/mL live *S aureus* were suspended in PBS and heat-inactivated by incubation in a water bath at 60^{o}C for 30 min. The heat-killed cells were then centrifuged at 2,000 ×g for 10 min and the supernatant discarded. The cells were then washed ×3 in PBS. The cells were re-suspended in PBS, stained with 120mM propidium iodide and then placed on an orbital shaker at 4^{o}C in the dark for 2 h. The PI-stained cells were then centrifuged at 2,000 g for 10 min, supernatant discarded and cells washed ×3 with HBSS (+0.1% gelatin). Human AB serum was denatured by incubation in water bath at 55 ^{o}C for 45 mins to inactivate the pathways (classical and alternative) of complement cascade. The heat-killed, PI-labelled *S. aureus* (SAPI) was then complement-opsonised by suspension in 30% (v/v) human AB serum (in PBS) and placed on an orbital shaker at 37^{o}C with gentle agitation for 1 h. The opsonised SAPI were then washed ×3 and re-suspended with PBS to a final concentration of 1×10^{9}/mL cells and stored at 4^{o}C.

2.12 Complement-opsonisation of polystyrene latex beads

For analysis of phagocytosis of latex beads by neutrophils and differentiated PLB-985 cells, 40µL of 10% suspension of 1.1 µm diameter non-fluorescent and amine-modified fluorescent polystyrene latex beads were re-suspended in 500 µL PBS and centrifuged at 5,000 ×g for 30 s. The supernatant was discarded and bead particles re-suspended in 500 µL. This was repeated ×2 to wash the beads. Following third wash, the beads were complement-opsonised by suspension in 190 µL PBS and addition of 10 µL heat-denatured human AB serum. These were mixed gently and incubated at room temperature for 2 h. The opsonised latex beads were then washed

×3 with PBS to remove unbound serum, and finally re-suspended in PBS to a final concentration of 1×10^9/mL bead particles.

2.13 Luminol-enhanced chemiluminescence assay of oxidative burst activity

The oxidative burst activity of neutrophils and differentiated PLB-985 cells was analysed by quantification of reactive oxygen species (ROS) produced in a luminol-enhanced chemiluminescence assay. Luminol readily diffuses through the cell membranes and rapidly becomes oxidised by free-radicals generated during the oxidative burst. The molecule then releases energy in the form of light, which was used to measure the total (intracellular and extracellular) oxidants generation.

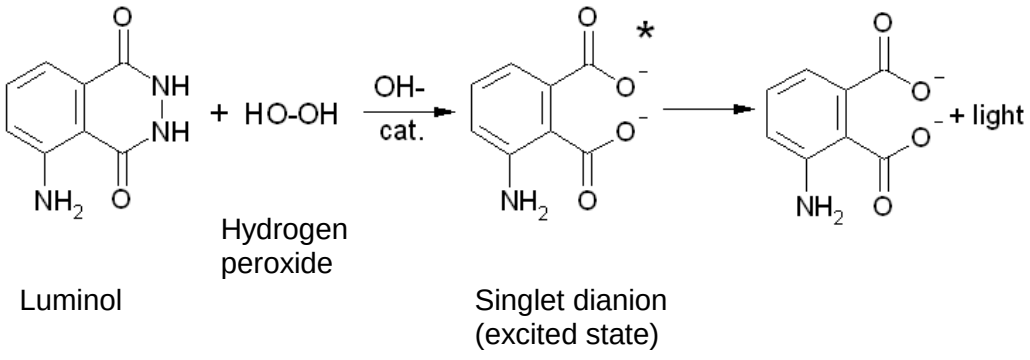


Figure 2.1 Luminol-enhanced chemiluminescence reaction. Reactive oxygen species (ROS) produced during oxidative burst activity oxidise the luminol into an electrochemically excited 3-aminophthalate intermediate, which emits energy in the form of photons of light as it returns to its ground state, measured as the total generated ROS using luminometer.

The chemotactic peptide, fMLP was used to stimulate receptor-dependent activation of the oxidative burst from the cells after incubation with GM-CSF, while receptor-independent activation was stimulated by PMA. Neutrophils (5×10^6 /mL) or dPLB-985 cells (2.5×10^6 /mL) were incubated in the absence and presence of G-CSF (10ng/mL) or GM-CSF (5 ng/mL) (for priming), at 37^{o}C for 45 min. Following incubations, 40 µL of cells was pipetted into wells in a white, low adhesion 96-well plate (as neutrophils produce a non-specific respiratory burst when adhered to TC plates) containing 100 µL HBSS media and 60 µL incubation mixture (158.8 µL HBSS media, 1 µL luminol (10 µM final concentration), and 1µM fMLP or 0.1µg/mL PMA final concentrations per well. Cells were pipetted as quickly as possible with PMA added first as its reaction is slow (5-10 min) followed by fMLP whose activation is very rapid (2-4 min). Both wells with unstimulated cells (no fMLP or PMA) and those with no cells (media alone) were also included to observe the differences in the background. The tubes and plates were wrapped in a thin foil during preparations because of the sensitivity of luminol to light. Chemiluminescence was measured as photon emission using a luminometer, and the total generated ROS was measured using a FLUOstar Omega plate reader from BMG Labtech set at 37^{o}C, 20% O_2, and 5% CO_2.

2.14 Flow cytometry

Flow cytometry analysis was performed with a Millipore 'Guava EasyCyte plus' flow cytometer. The technique measures single cells in a population of cells and quantifies them according to their light scattering properties or their surface or internal fluorescence following staining with fluorescent dyes or labelled antibodies. The method involves passage of cell suspensions through a single wavelength laser beam. The passage of cells scatters the laser-light which is then quantified by two

detectors; the parallel detector which measures the forward scatter (FSC) indicative of cell size and the perpendicular detector, which analyses the side scatter (SSC) indicative of cell granulation. Alternatively, fluorophores on cells stained with labelled antibodies become excited by the laser beam and emit fluorescence which is measured as the cell's expression of a particular component. Samples were analysed in a 96-well plate. The distributions of fluorescence represent 5,000 gated events analysed per sample.

2.14.1 Flow cytometry analysis of cell viability

The Guava viaCount assay was used to measure cell viability. The Guava viaCount reagent contains two DNA binding dyes: nuclear and viability dyes. The assay distinguishes viable and dead/apoptotic cells based on differential permeability of the two dyes. The nuclear dye is cell-permeable and stains DNA in all cells and the viability dye is cell-impermeable and only stains DNA and RNA in dying cells with a permeabilised cell membrane. Following appropriate incubations in culture media, 25 µL of cells (neutrophils at 5×10^6 /mL and PLB-985 at 1×10^6 /mL) was pipetted into relevant wells in a 96-well plate, containing 225 µL of ViaCount reagent (1:10 dilution) and incubated for 5 min at room temperature. The cell viability was then analysed using Guava ViaCount software on flow cytometer. 5,000 gated events were analysed per sample.

2.14.2 Flow cytometry analysis of neutrophil apoptosis

The phospholipid bilayer of the plasma membrane contains phosphatidylserine groups which in healthy cells, are restricted to the cytoplasmic side of the membrane, but when the cells enter apoptosis, these became delocalised and appear on the cell

surface. Phagocytes recognise and remove apoptotic cells *in vivo* by these cell surface phosphatidylserine groups. A cellular protein Annexin V, whose function is unknown, binds the phosphatidylserine groups on the cell surface (Crowley et al., 2016). Therefore, FITC-conjugated Annexin V binds the cell surface phosphatidylserine groups of apoptotic cells and produces fluorescence which is measured as an indicator of an early stage apoptosis. Late-stage apoptosis/necrosis on the other hand, is determined using propidium iodide (PI) as an indicator. Propidium iodide is impermeable to the plasma membrane and is therefore, prevented entry into viable cells. However, apoptosis renders the plasma membrane permeable thus, allowing propidium iodide to enter the cell and bind to DNA, leading to an enhanced fluorescence (Rieger et al., 2011). Usually, an 18 h incubation of neutrophils resulted in 60-70% apoptosis in cells not treated with any apoptosis delaying-agent.

1mL of cells at 10^6 /mL in RPMI + 10% human AB serum was incubated per well in a 24-well plate, for 0, 2, 4, 6 h and overnight at 37^{o}C in a humidified atmosphere of 5% CO_2. 1 µL G-CSF (10 ng/mL) or GM-CSF (5 ng/mL) was used as a positive control for delayed apoptosis. Following incubation, samples were gently pipetted up and down wells several times to re-suspend the cells and then 25 µL was removed to a 96 wells plate. To stain the cells, 25 µL HBSS containing 0.5 µL Annexin V was added to each well and incubated for 15 min at room temperature in the dark. This was followed by the addition of 200 µL HBSS containing 1 µg/mL propidium iodide. The samples were then analysed immediately on the flow cytometer, and 5,000 gated events were analysed per sample.

2.14.3 Flow cytometry analysis of cell cycle and DNA staining

The cell cycle parameters of neutrophils and PLB-985 cells were analysed by flow cytometry, using univariate approach that measures total cellular DNA content and reveals the distribution of cells in three major phases of the cell cycle (G1, S and G2/M), after staining permeabilised, fixed cells with propidium iodide. It also allows detection of apoptotic cells in the dormant phase (G0) with fractional DNA content. Propidium iodide binds DNA and produces fluorescence which is proportional to the DNA concentration per cell, and which varies as the cells progress through the cell cycle.

200 µL of cells at 10^6 /mL were pipetted into a small microfuge tube after appropriate incubations as described in the text, and centrifuged at 1,000 g for 3 min. The supernatant was discarded, the pellet flicked and washed by re-suspension in 200 µL PBS (+EDTA) and centrifuged at 1,000 g for 3 min. The supernatant was discarded and the pellet flicked and re-suspended in 200 µL ice-cold, 70% ethanol and kept at 4ºC for at least 1 h or overnight (when fluorescence was not to be measured immediately) to fix the cells. Triton-X/RNase/PI staining buffer (1 mg/mL PI in PBS and 1 mg/mL Ribonuclease A in 1% Triton/PBS combined in 1:9 ratio) was prepared just prior to analysis and kept in the dark as PI is light sensitive. The fixed cells were centrifuged at 1,000 g for 3 min, supernatant aspirated, pellet re-suspended in 200 µL PBS (+EDTA) and centrifuged at 1,000 g for 3 min to remove the ethanol before staining. The supernatant was aspirated and the pellet re-suspended in 200 µL PBS (+EDTA). 200 µL of fixed (ethanol-free) cells was then transferred into wells in 96-well plate containing 25 µL of the staining buffer. The cells were analysed immediately using the flow cytometer, and 5,000 gated events were analysed per sample.

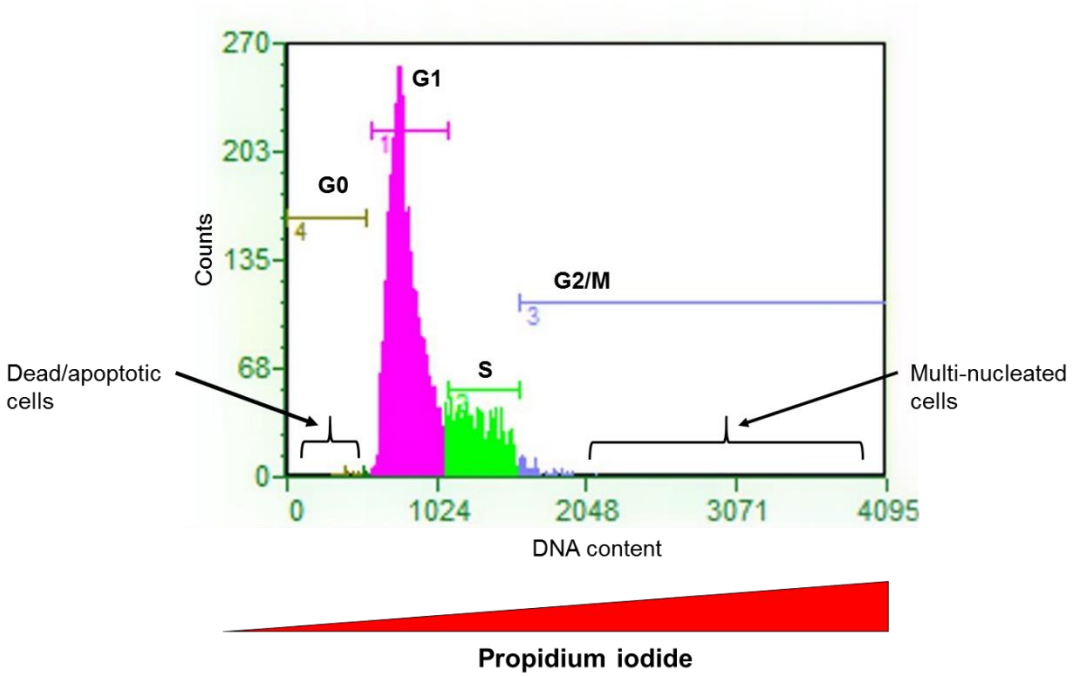


Figure 2.2 Typical flow cytometer trace of DNA distribution profile indicating cell cycle parameters. The non-synchronously growing PLB-985 cells were permeabilised, fixed and stained with propidium iodide. The PI binding intensity increases as the cell progresses through the cell cycle and the amount of PI fluorescence is proportional to the increasing DNA concentration.

2.14.4 Flow cytometry analysis of cell surface receptors expression

To study the expression of cell surface receptors (CD11b, CD14, and CD16) on the surface of neutrophils and differentiated PLB-985 cells, 10 µL of cells (neutrophils at $5x10^6$/mL and PLB-985 cells at $2.5x10^6$/mL) were pipetted into a small microfuge tube, diluted with 200 µL PBS (+BSA) and centrifuged at 900 g for 3 min. The supernatant was discarded, tubes flicked and the pellet re-suspended in 10 µL PBS (+BSA). Appropriate (saturating) antibody volumes optimized for flow cytometry (as supplied by the manufacturers) were added as described in Table 2.3 and mixed by votexing. Cells were incubated for 30 min at 4^{o}C in the dark. 200 µL PBS (+BSA) was then added and centrifuged at 900 g for 3 min. Supernatant was discarded and cells fixed by addition of 100 µL PBS (+BSA) and an equal volume of 4% paraformaldehyde (PFA) at room temperature, in the dark for 15 min. Fixed cells were then centrifuged at 900 g for 3 min, supernatant discarded and pellet re-suspended in 200 µL PBS (+BSA). Cells were then analysed for expression of the surface antigens using the flow cytometer or stored overnight in the fridge (in the dark) if the analysis was not to be done immediately.

2.14.5 Permeabilisation of cells and intracellular CD16 staining

For intracellular CD16 staining, cells were permeabilised using 0.1% saponin (in PBS) prior to staining. Following fixation with 4% PFA and washing, cells were suspended in 200 µL PBS (+ 0.1% saponin) and incubated at 4^{o}C for 15 min, centrifuged at 900 g for 3 min, and supernatant discarded. Cells were then re-suspended in 10 µL PBS (+ 0.1% saponin) and 2 µL CD16 added and incubated at 4^{o}C for 30 min. Cells were then washed to remove excess/unbound antibody by suspension in 200 µL PBS (+BSA), centrifuged at 900 g for 3 min, supernatant discarded and pellet re-

suspended in 200 µL PBS (+BSA). Expression of intracellular CD16 was analysed by flow cytometry.

Table 2.3 Conjugated antibodies used in flow cytometry

Antibody	**Neutrophil concentration**	**dPLB-985 cells concentration**	**Antibody volume**
CD11b-FITC CD11b-PE CD14-PerCP	5 x 10^6 in 10 µL	2.5 x 10^4 in 10 µL	5 µL
CD16-FITC	5 x 10^6 in 10 µL	2.5 x 10^4 in 10 µL	1 µL

2.14.6 Flow cytometry analysis of phagocytosis

Neutrophils or PLB-985 cells at 1×10^6/mL were incubated at 37^{o}C in the presence or absence of priming agents; G-CSF (10ng/mL) or GM-CSF (5 ng/mL) for 30 min. Complement-opsonised SAPI or latex beads at 1:10 (cell to particle ratios) were added to the incubation mixture with gentle agitation on an orbital shaker in a 5% CO_2 incubator at 37^{o}C for a further 30 min. Unstained or unstimulated cells were included to gate for negative fluorescence. The samples were centrifuged at 1,000 x g for 3 min, the supernatant discarded and cells re-suspended in 1mL HBSS or PBS. 200 µL of the cell suspension was then pipetted into wells in a 96-well microtitre plate. The phagocytosis of SAPI or latex beads by neutrophils and dPLB-985 cells was measured by flow cytometry, analysing 5,000 gated events per well.

2.14.7 Flow cytometry analysis of oxidative burst activity

Neutrophils or PLB-985 cells at 1×10^6/mL were incubated at 37^{o}C in the presence or absence of priming agents; G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of DHR 123 (5µM) for 15 min, as a ROS detection probe. Oxidative burst activity was then stimulated by addition of fMLP (1µM) for 5 min, and PMA (0.1µg/mL) or latex beads at 1:10 (cell to particle ratios) for 15 min, including samples containing DHR 123 alone, as well as unstimulated cells to gate for negative fluorescence. The samples were then centrifuged at 1,000 x g for 3 min, the supernatant discarded and cells re-suspended in 200 µL HBSS or PBS and oxidative burst activity of neutrophils or differentiated PLB-985 cells was measured immediately by flow cytometry. The distributions of fluorescence represented 5,000 gated events analysed per sample.

2.15 Transmigration and chemotaxis assay

Millipore hanging cell culture plate inserts were used to measure the transmigration of neutrophils and differentiated PLB-985 cells. 24-well tissue culture plates were coated with poly-hema (2-hydroxymethyl methacrylate) at a concentration of 12 mg/mL in ethanol to prevent cells adhering to the plates following transmigration, and used for the assay. The plates were incubated at 37^{o}C, 5% CO_2, overnight or until the ethanol evaporated. 800 µL RPMI 1640 (+10mM L-glutamine) media without and with chemoattractants; fMLP (10^{-8}M), IL-8 (0.1µg/mL), zymosan A (1µg/mL) or casein (0.5 mg/mL) were added into the relevant wells. The hanging inserts fitted with a 3 µm pore sized filters at their bases were suspended in the media and left for at least 10 min at room temperature to equilibrate.

$1x10^6$ neutrophils or 4×10^5 dPLB-985 cells in 200 µL media were added into the hanging inserts and incubated at 37°C, 5% CO_2 for 90 min. The hanging inserts were then removed from the wells and the cells that transmigrated into each well were counted using Multisizer 3 cell coulter counter (Beckman Coulter) following a 1:1000 dilution with Isoton II. The number of migrated cells was expressed as a percentage of total number of cells added originally.

2.16 Statistical analysis

Statistical analysis was performed using IBM SPSS v20 software. Student's t-test (independent and paired) or one-way analysis of variance (ANOVA) were used to analyse the data following testing for normality. Differences were considered significant within 5% confidence interval ($p\leq0.05$) and the values were denoted by *. Error bars displayed in graphs signify the mean ± SEM where repeat experiments were performed. Some error bars are however, undetectable or appear extremely small due to low error in the affected data sets.

Chapter 3: Differentiation of PLB-985 cell line into mature neutrophil-like granulocytes

3.1 Introduction

In 1971, Friend induced the murine erythroid leukaemia (MEL) cells to differentiate into orthochromic erythroblasts *in vitro* using dimethylsulfoxide (DMSO). This was the first demonstration of *in vitro* differentiation of leukaemia cell lines (reviewed in Watanabe et al., 1988). Following this, wide range of chemical agents have been demonstrated to induce *in vitro* differentiation of leukaemia cell lines into more mature phenotypes which include hexamethylenebiacetamide (HMBA), ester 12-O-tetradecanoylphorbol-13-acetate (TPA) (Ji-wang et al., 2000), *N,N*-dimethylformamide (DMF) (Katschinski et al., 1999), retinoyl glucuronide (Zile et al., 1987) and *all trans* retinoic acid (ATRA) (Trayner et al., 1998). Likewise, many cell lines with the capacity to differentiate along the granulocytic pathway have also been established, such as U-937 in 1974 (Sundström & Nilsson, 1976, Harris & Ralph, 1985), K-562 in 1975 (Andersson et al., 1979, Klein et al., 1976), HL-60 in 1977 (Collins, 1987), KG-1 in 1978 (Koeffler & Golde, 1978) and PLB-985 in 1985 (Tucker et al., 1987).

Phenotypically, the human promyelocytic leukaemia PLB-985 cell lines are myelomonoblast-like cells, and upon exposure to appropriate inducing agents, they can be induced to differentiate into more mature granulocytes or monocytes/macrophages depending on the inducing-agent (Shin & Demura, 2012). Chemical agents, such as dimethylsulfoxide (DMSO) (Shin & Demura, 2012), dimethylformamide (DMF) (Katschinski et al., 1999), dibutyrylcyclic AMP (dbcAMP) (Hazan-Eitan et al., 2006) and granulocyte colony-stimulating factor (G-CSF) (Kim &

Seoh, 2015) have been described to induce PLB-985 cell line to differentiate through the granulocytic pathway into mature neutrophil-like cells. These cells have also been induced to differentiate into monocytes/macrophages after incubation with phorbol myristate acetate (PMA) (Perkins et al., 1991), 1,25-dihydroxy vitamin D3 (Perkins et al., 1991) and interferon-y (Hazan-Eitan et al., 2006). In the present study, mixture of *all-trans* retinoic acid (ATRA), *N, N*-dimethylformamide (DMF) and sodium pyruvate was used to induce differentiation of PLB-985 cells into mature neutrophil-like granulocytes.

Retinoic acid (RA) is one of the most potent differentiation-agents that induces *in vitro* differentiation of leukaemia cell lines. The use of retinoic acid to induce *in vitro* differentiation can be traced back to 1978 when Collins *et al.,* used two isomers of retinoic acid; all-*trans* retinoic acid (ATRA) and 13-cis retinoic acid to induce the human promyelocytic leukaemia HL-60 cell line to differentiate into mature neutrophil-like cells (Dong et al., 2003, Collins et al., 1978). Retinoic acid had been shown to induce both morphological and functional differentiation of cultured HL-60 cells, in contrast to DMSO which induced only their morphological differentiation (Martin et al., 1990). Terminal differentiation of leukaemia cell lines induced with ATRA was found to be followed by a spontaneous apoptosis (Degos & Wang, 2001).

N, N-dimethylformamide (DMF) is another chemical agent with differentiation and anti-tumour activity. DMF and its metabolite *N*-methylformamide (NMF) are capable of inducing *in vitro* differentiation of leukaemia cell lines into mature phenotypes (van Dongen et al., 1989). It has been used to induce erythroleukaemia cells to differentiate into erythocyte-like cells (Scher et al., 1973) and has been demonstrated to stimulate differentiation of HL-60 into mature granulocytes (Collins et al., 1978). Katschinkski *et al.* (1999) reported induction of PLB-985 cells into granulocytic differentiation using dimethylformamide (Katschinski et al., 1999).

Sodium pyruvate is a metabolite that serves as an additional carbon or energy source and also a free-radical scavenger that eliminates hydrogen peroxide generated in the culture media, thereby substantially protecting the cells against reactive oxygen intermediates induced toxicity (Giandomenico et al., 1997). As an anti-oxidant, pyruvate also stabilizes ATRA by protecting it from being oxidised. It has been reported to be effective in protecting the human neuroblastoma cell line SK-N-MC against hydrogen peroxide induced apoptosis through inhibition of caspase 3 activity (Jagtap et al., 2003). It has also been shown to promote *in vitro* nuclear maturation of bovine oocytes (Geshi et al., 2000).

In this Chapter, PLB-985 cells were cultured in the presence of three differentiation- and maturation-inducing agents namely; *all trans* retinoic acid (ATRA), *N, N*-dimethyformamide (DMF) and sodium pyruvate in order to induce their full differentiation into mature neutrophil-like cells.

Therefore, the aims of this Chapter were to:

1. Optimise the culture conditions that induce immature promyelocytic leukaemia PLB-985 cell lines to terminally-differentiate into mature neutrophil-like granulocytes.
2. Determine the time course of differentiation of PLB-985 cells into mature neutrophil-like cells.
3. Identify whether the terminally-differentiated PLB-985 cells resemble mature blood neutrophils phenotypically.

3.2 Methods

PLB-985 cells were cultured with a starting density of 2×10^5/mL. ATRA (1μM), DMF (0.5% (v/v)) and sodium pyruvate (1% (v/v)) were added to the media to induce differentiation. The initial experiments carried out were to establish a protocol for successful induction of differentiation of PLB-985 cells into neutrophil-like phenotypes. In preliminary experiments, cells were cultured in unchanged media for 6-7 days (in control- and differentiation-media) and their growth rate measured by cell counts. Subsequently, cells were re-suspended in fresh differentiation media after day 2 and 4 of culture, in order to enhance their proliferation, differentiation and survival. To determine if the experimental conditions could be further modified for maximum differentiation and survival, proinflammatory cytokines, G-CSF or GM-CSF and Toll-like receptors (TLRs) agonists (LPS, MALP-2, Pam3CSK4 and R848) were also added to the cell cultures during the media changes at days 2 and 4, and their effects in stimulating proliferation, differentiation and survival of granulocytes were measured.

3.3 Results

3.3.1 Growth and differentiation of PLB-985 cells

In initial experiments PLB-985 cells were cultured in the absence and presence of differentiating agents (as described in Methods), and total cell counts were measured using Multisizer3 cell counter, as well as numbers of differentiated and apoptotic cells by microscopy and flow cytometry during culture for up to 6/7 days. The routine media for the growth and maintenance of PLB-985 cells is RPMI-1640 medium,

supplemented with 10mM L-glutamine, 10% (v/v) foetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin (P/S). Initial experiments aimed at inducing differentiation used this medium except that it contained only 0.5% (v/v) foetal calf serum, and was supplemented with 1µM *All-trans* retinoic acid (ATRA), 0.5% (v/v) *N, N*-dimethylformamide (DMF) and 1% (v/v) sodium pyruvate. The routine medium (designated as PLB-985) was therefore used in parallel with this modified differentiation medium (designated as dPLB-985) and cells were cultured in both media for a period of 7 days. Each day, samples were removed for cell counts, number of viable cells and number of differentiated cells measurements.

The modified differentiation medium successfully induced the differentiation of PLB-985 cells into neutrophil-like phenotypes. However, several experimental conditions have been employed to optimise the differentiation efficiency and prolong the survival of the differentiated cells, described in the proceeding sections.

3.3.1.1 Differentiation conditions 1

The PLB-985 cells cultured under the two conditions (PLB-985 and dPLB-985) both showed an exponential growth pattern with a doubling time of approximately 24 h (Figure 3.1) during first few days of the culture (days 1-3). Thereafter, the proliferation rate declined as the cells aged in the culture. By day 3, the cell number was lower in the dPLB-985 cultures due to the loss of proliferation capacity caused by ATRA (Degos & Wang, 2001), DMF (Van Dongen et al., 1989) and sodium pyruvate (Long & Halliwell, 2009). The numbers of cells in both PLB-985 and dPLB-985 cultures were counted using a Multisizer3 cell counter daily or on selected days during the culture period, while the cell morphology was examined by light microscopy after preparation

of cytospin slides and Romanowsky staining. These parameters were used to confirm proliferation and morphological changes associated with differentiation. However, as the cell culture period extended beyond 3 days, the rate of cell proliferation declined and this was most marked for the dPLB-985 cells (Figure 3.1). In both cell cultures numbers declines by day 6 and 7 (Figure 3.1)

At time points throughout this culture, samples were removed for analysis of cell morphology by Romanowsky staining of cytospin slides and light microscopic examination to determine cell viability and morphology. Differentiation was initially assessed by morphological criteria and compared to the morphology of mature, blood neutrophils (Figure 3.2). These cells are characterised by their relatively small cell size (~ 8-12 µm diameter) compared to non-differentiated PLB-985 cells, multi-lobular nucleus and granulated cytoplasm. These parameters were used to confirm the morphological changes associated with differentiation of PLB-985 cells.

PLB-985 cells cultured in non-differentiation medium (PLB-985) had a round, diffuse nucleus that occupied a large proportion of the cytoplasm. This nucleus was generally rounded in morphology (Figure 3.3A) and no morphological features of differentiation were observed at any time points during the 7-day culture. When PLB-985 cells were cultured in differentiation medium (dPLB-985), they began to show signs of differentiation, such as indentations and convolutions in the nuclear membrane after day 2 of differentiation, and by day 5-6, many cells had acquired morphological features of a mature neutrophil, such as decreased cell size, segmentation of the nuclei into 3-5 lobes, disappearance or decreased appearance of nucleoli and granulation of the cytoplasm (Figure 3.3B). However, some cells showed morphological features of changes in nuclear morphology, but the nucleus could not be termed multi-lobed (Figure 3.3B (i)). Instead, these cells resembled band cells (not fully-matured neutrophils) and hence in this thesis, such cells are defined as “partially

differentiated". Some cells showed clearly-defined multi-lobed nuclei (Figure 3.3B (ii)), and hence these were defined as "differentiated cells". Some cells, however, showed few, if any signs of differentiation as assessed by nuclear morphology (Figure 3.3B (iii)) and were termed "non-differentiated cells".

These morphological changes that indicate differentiation towards neutrophils had occurred were evident from day 3 in culture and by day 4, >70% of the cells (Table 3.1) showed signs of differentiation, with ~ 60% having a fully-defined multi-lobed nucleus that is a defining characteristic of mature neutrophil-like cells. After day 5, a mixture of different cell populations was observed, resulting from various stages of differentiation undergone by the cells (Figure 3.3B). These include undifferentiated cells (6%), band cells (14%), differentiated cells (60%) and apoptotic/dead cells (20%).

However, after day 4 of culture in dPLB-985, there were cells clearly identified in cytoslide spins that showed signs of apoptosis (Figure 3.3B (iv)), and these increased in number over the following days (Table 3.1). These were defined as small, round cells with a highly-condensed, round nucleus. Few, if any apoptotic cells were observed in the undifferentiated cultures at any time points. This suggested that after the PLB-985 cells had differentiated into mature neutrophils, they underwent apoptosis. This rapid transition into apoptosis is a characteristic of mature blood neutrophils, which after isolation and culture *in vitro* for 18-24 h (in the absence of any anti-apoptotic agent), approximately 60-70% will become apoptotic (Derouet et al., 2004). Therefore, this culture and differentiation protocol was modified with the aims of trying to (a) increase the percentage of fully-differentiated cells and (b) to decrease the rate of apoptosis once the cells had differentiated into mature cells. Representative cytospin slide images of dPLB-985 cells cultured in differentiation condition 1 for each day over 7 days period are shown in Figure 3.4.

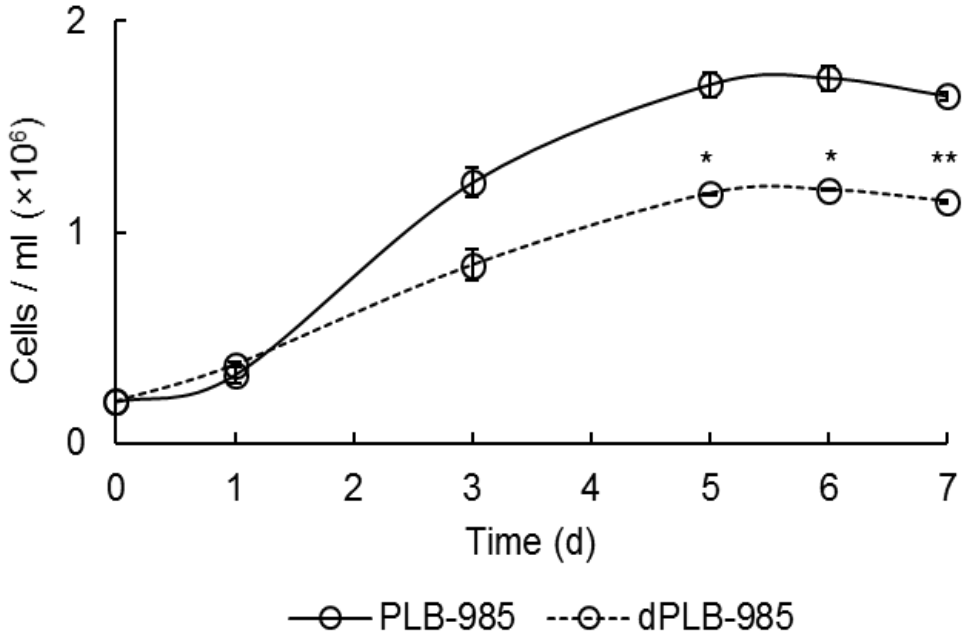


Figure 3.1 Growth of PLB-985 cells in the absence and presence of differentiation-inducing agents. Exponentially growing PLB-985 cells at a starting density of 2×10^5/mL were cultured in separate flasks designated PLB-985 (non-differentiating) and dPLB-985 (differentiation-induced) cells. The media was kept unchanged for 7 days and cell number counted on days 0, 3, 5, 6 and 7 using a Multisizer 3 coulter counter, following 1:1000 dilution with Isoton II. The cells had a doubling time of 24 h initially, but after day 3, the growth rate decreased, owing to neutrients depletion and perhaps death by apoptosis, particularly in dPLB culture. Data are expressed as mean of total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

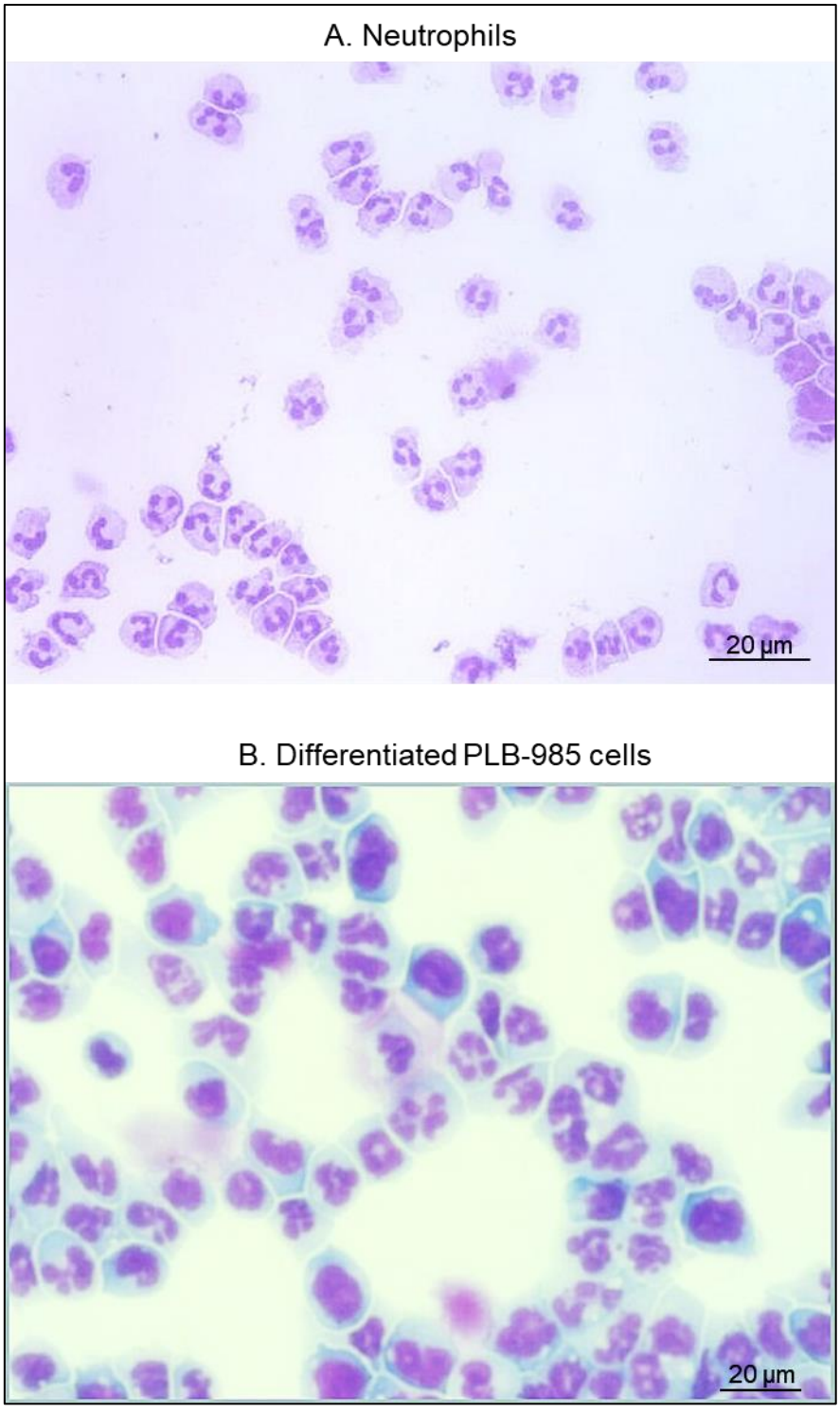


Figure 3.2 Morphology of blood neutrophils and dPLB-985 cells. Freshly-isolated blood neutrophils (A) and neutrophil-like differentiated PLB-985 cells at day 5 of culture (B) were prepared, cytocentrifuged, stained with rapid Romanowsky stain and viewed under a light microscope. The prominent multi-lobed nuclei and granulated cytoplasms were evident in both cell types.

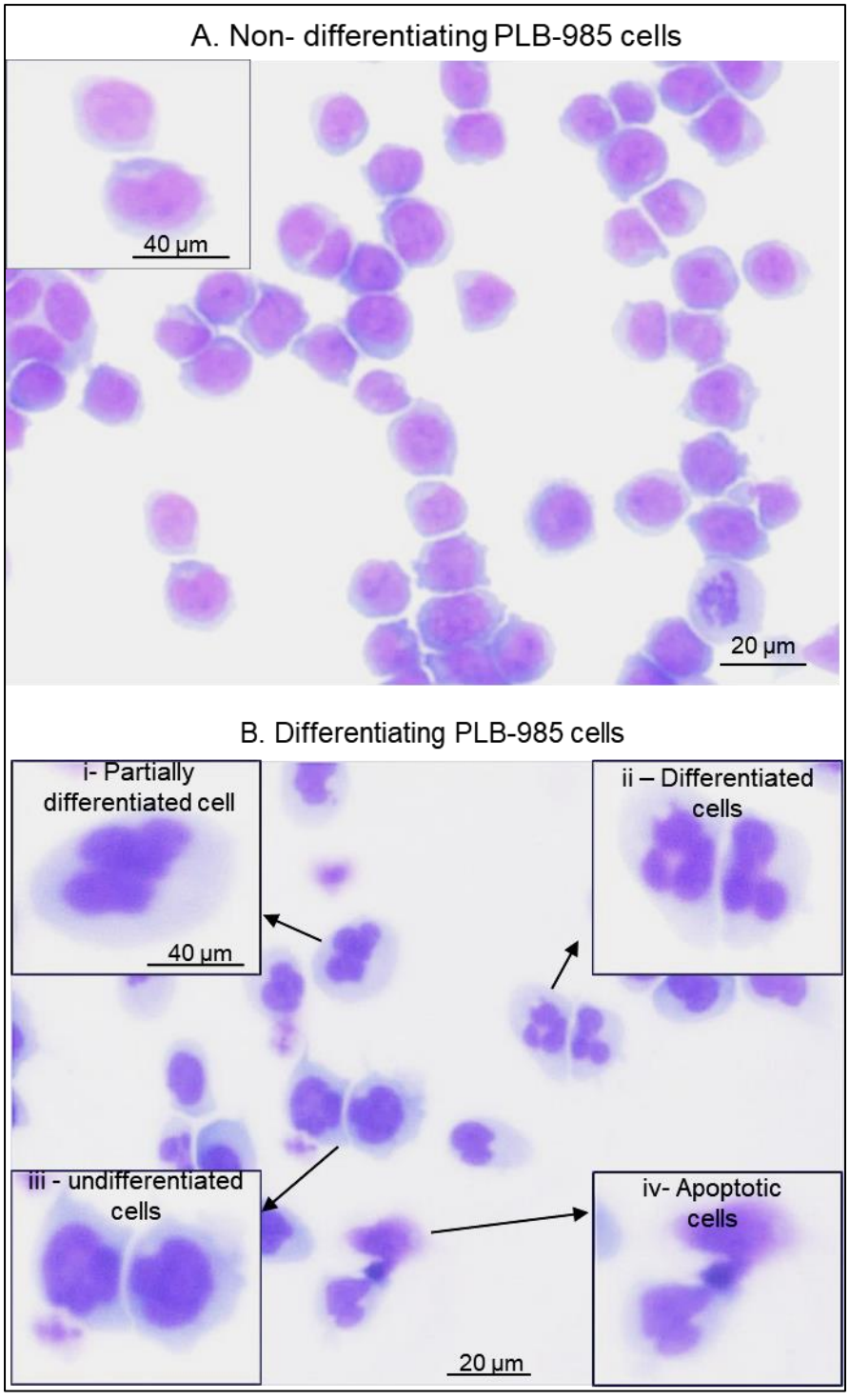


Figure 3.3 Morphological changes during differentiation of PLB-985 cells. PLB-985 cells were cultured in the absence (A) and presence (B) of differentiation inducing agents. Cells samples were removed on day 5, cytospin slides prepared, stained with Romanowsky stain and viewed under the light microscope. In B, insets show (i) a partially differentiated (band) cell, (ii) fully differentiated cells, (iii) non-differentiated cells and (iv) apoptotic/dead cells.

Table 3.1 Percent proportions of dPLB-985 cells after day 3-6 of induction.

Cells Differentiation Status	Day 3	Day 5	Day 6
Differentiated (%)	44.41 ± 4.07	65.89 ± 5.17	59.33 ± 4.39
Partially differentiated (%)	24.74 ± 4.32	13.35 ± 2.04	10.94 ± 4.24
Non-differentiated (%)	31.44 ± 3.54	8.41 ± 4.16	6.21 ± 3.36
Apoptotic (%)	4.41 ± 1.36	12.35 ± 3.61	23.52 ± 4.43
Total	100	100	100

Proportions of differentiation of PLB-985 cells counted manually by morphological examination of cells from representative cytospin slides viewed under the light microscope, after day 3 of culture when cells began to exhibit vividly clear features. Counts expressed as percentages of the total number of cells (± SD, n=3).

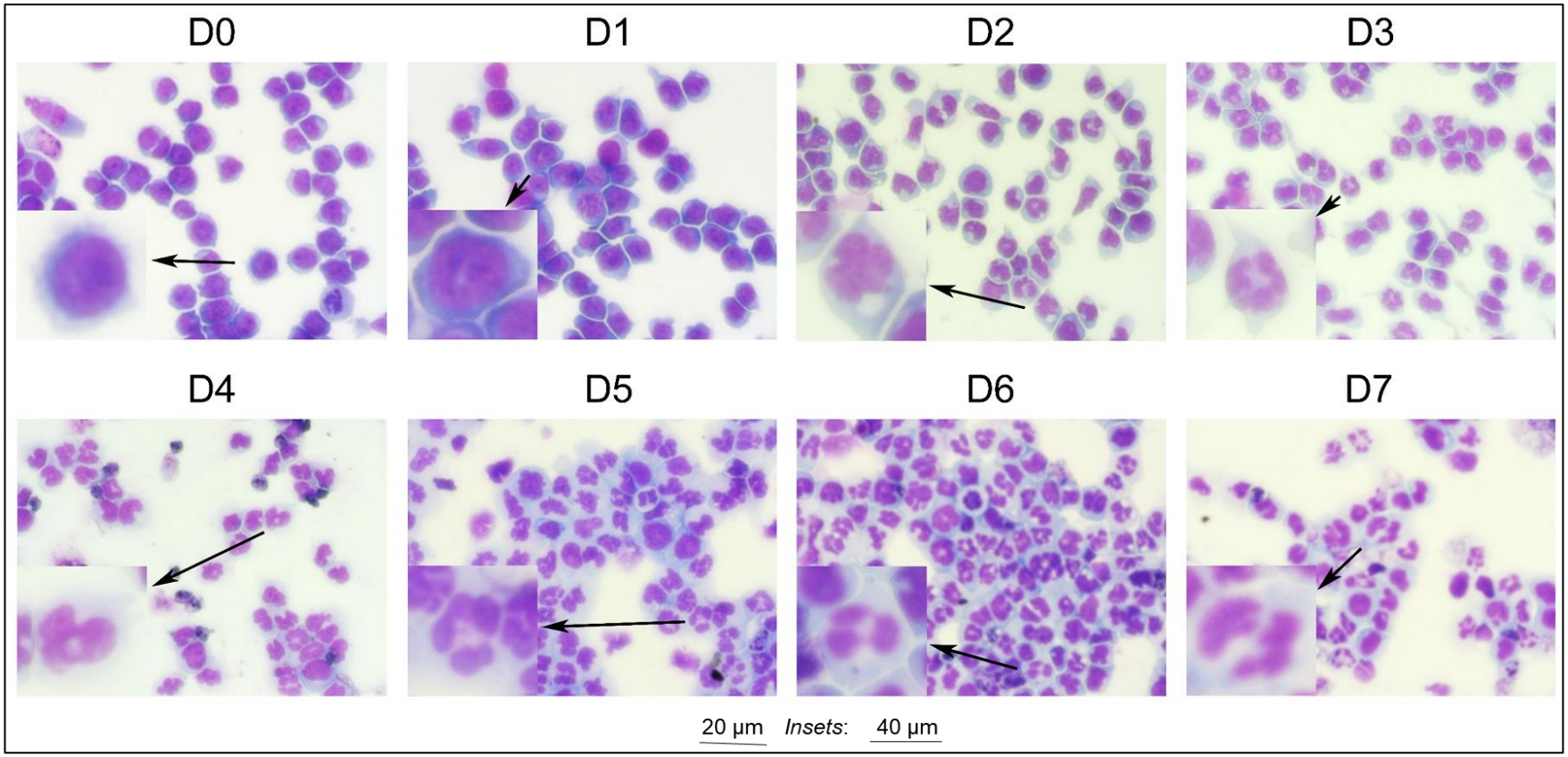


Figure 3.4 Representative cytospin slides images of dPLB-985 cells morphology. dPLB-985 cells were cultured in the presence of differentiation-inducing agents for 7 days and cell samples were removed, cytospin slides prepared, stained with Romanowsky stain and viewed under the light microscope daily. Typical large, non-lobular nuclei, frequent nucleoli and agranular cytoplasm were evident on day 0-1. After day 2 of induction, cells began to show signs of differentiation, such as indentations in the nucleus, decrease in number of nucleoli, and appearance of granules in the cytoplasm. By days 4-7, the cells had acquired multi-lobular nuclear morphology. Some cells exhibiting apoptotic morphologies were also evident from day 4.

3.3.1.2 Differentiation conditions 2

The previous differentiation conditions successfully induced differentiation of PLB-985 cells into mature neutrophil like phenotypes, but improvements in these culture conditions were then explored in order to increase the efficiency of differentiation of PLB-985 cells and to enhance the survival of the differentiated cells. To achieve these goals, several variations in the above differentiation protocol were explored. First, the differentiation culture medium was changed twice (at day 2 and 4) during the 7-day culture period. The rationale behind this approach was that, in the absence of a medium change over 7 days of culture, nutrient depletion and changes in pH of the media, as well as accumulation of excretory waste products, may contribute to decreased growth, impairment of differentiation and acceleration of apoptosis.

3.3.1.2.1 Effect of media changes on differentiation and viability of dPLB-985 cells

The effects of differentiation media changes on days 2 and 4 of the culture period, on the percentage of differentiated PLB-985 cells are shown in Figure 3.5A. As can be seen, changing the culture medium in this way resulted in a significantly increased percentage of differentiated cells, compared to cells that were cultured for 7 days continuously in unchanged medium. In these experiments, cell viability was also measured using the Viacount assay (see Chapter 2, methods), which measures changes in the permeability of the plasma membrane. In the absence of changes in the culture medium at days 2 and 4, the viability of the differentiated cells, which was close to 100% over the first two days of culture, gradually decreased to around 20% by day 5 and then remained at around this level up to day 7 (Figure 3.5B). However, in differentiated cultures in which the media was replaced with fresh media at days 2

and 4, there was a statistically-significant ($p<0.01$, $n=3$) increase in the viability from day 4 onwards, compared to cells without media changes. By day 6, ~60% of the cells were viable after the media changes, whereas only ~20% were viable in the absence of these media changes.

Superimposed plots for percentages of differentiation and viability of the cells cultured under these two different conditions is shown in Figure 3.6. Representative cytospin slide images showing these changes in morphology from day 3-6 in cultures without the media changes (NMC) and with the media changes at days 2 and 4 (MC- D2, D4) are shown in Figure 3.7.

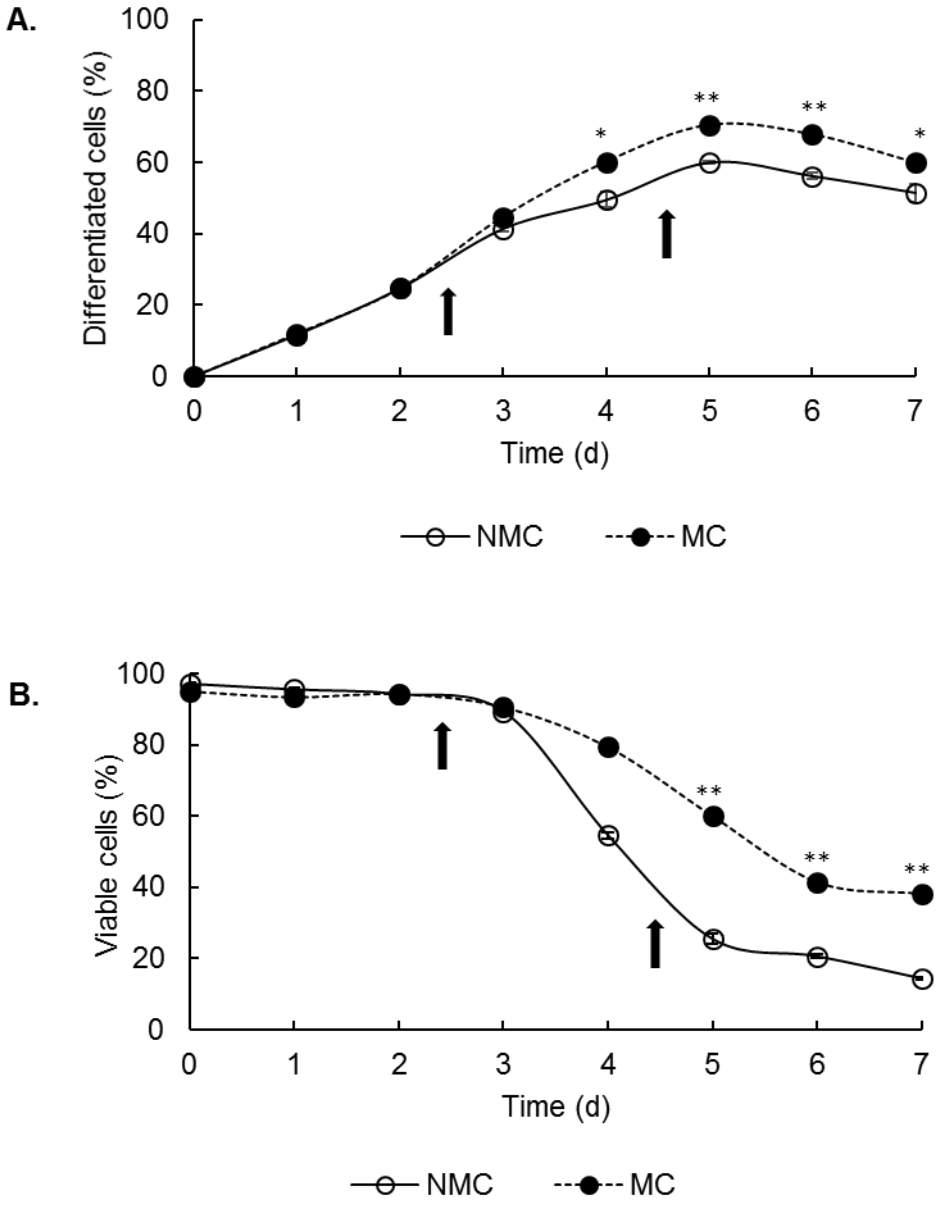


Figure 3.5 Effect of media changes on differentiation and viability of dPLB-985 cells. PLB-985 cells were cultured in differentiation media for 7 days, without (NMC) and with media changes on days 2 and 4 (MC). The number of differentiated cells was counted manually from representative cytospin slides viewed under the light microscope and the viability was measured using the viaCount assay on the flow cytometer. (A) Shows the number of differentiated PLB-985 cells and (B) shows dPLB-985 cells viability. Arrows indicate times of media changes and data are expressed as percentage of total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

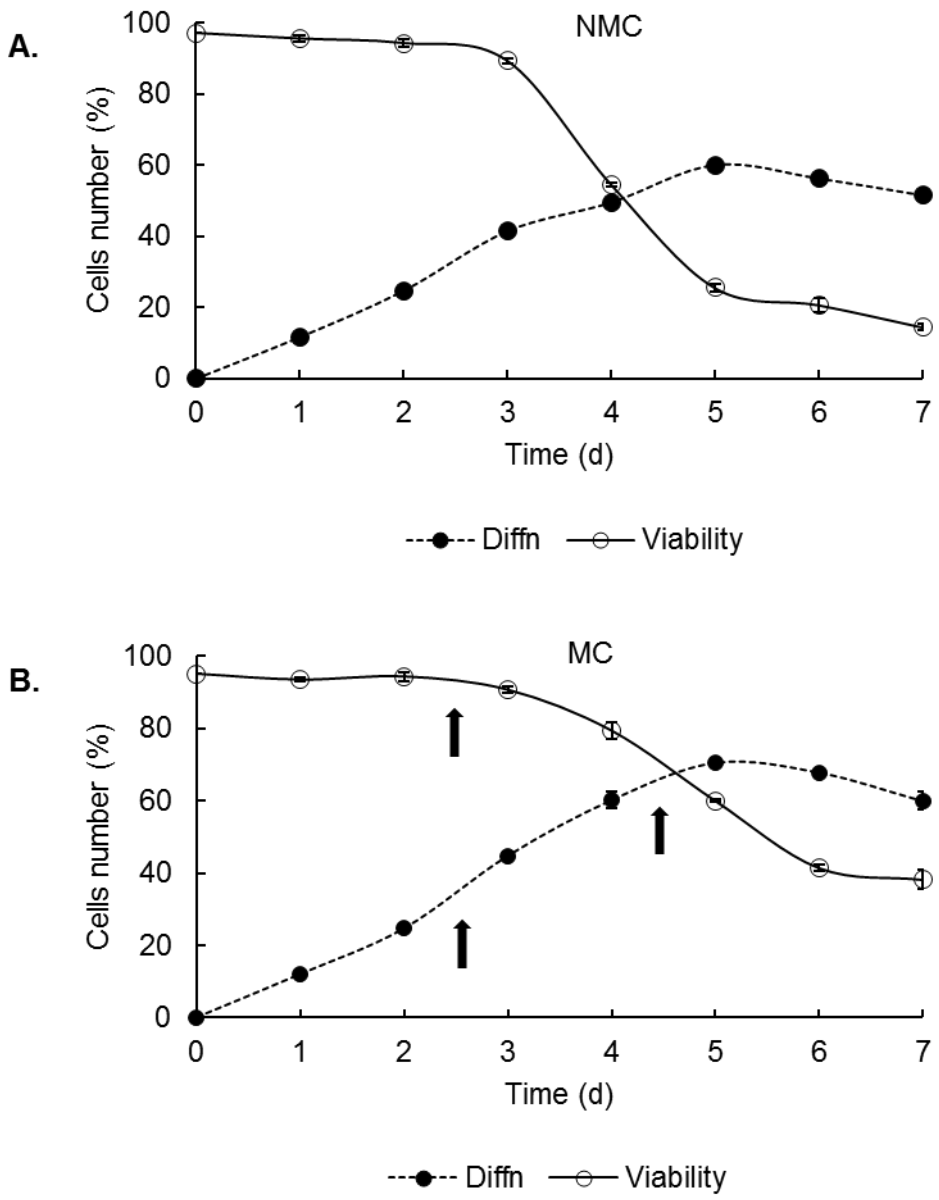


Figure 3.6 Superimposed plots of differentiation versus viability of dPLB-985 cells. PLB-985 cells were cultured in differentiation media for 7 days, without (A) and with (B) media changes on days 2 and 4. The number of differentiated cells was counted manually from representative cytospin slides viewed under the light microscope and the viability was measured using the viaCount assay on the flow cytometer. Arrows indicate times of media changes and data was expressed as percentage of total cells. Most of the differentiated cells in (A) died after day 4 of culture due to spontaneous apoptosis.

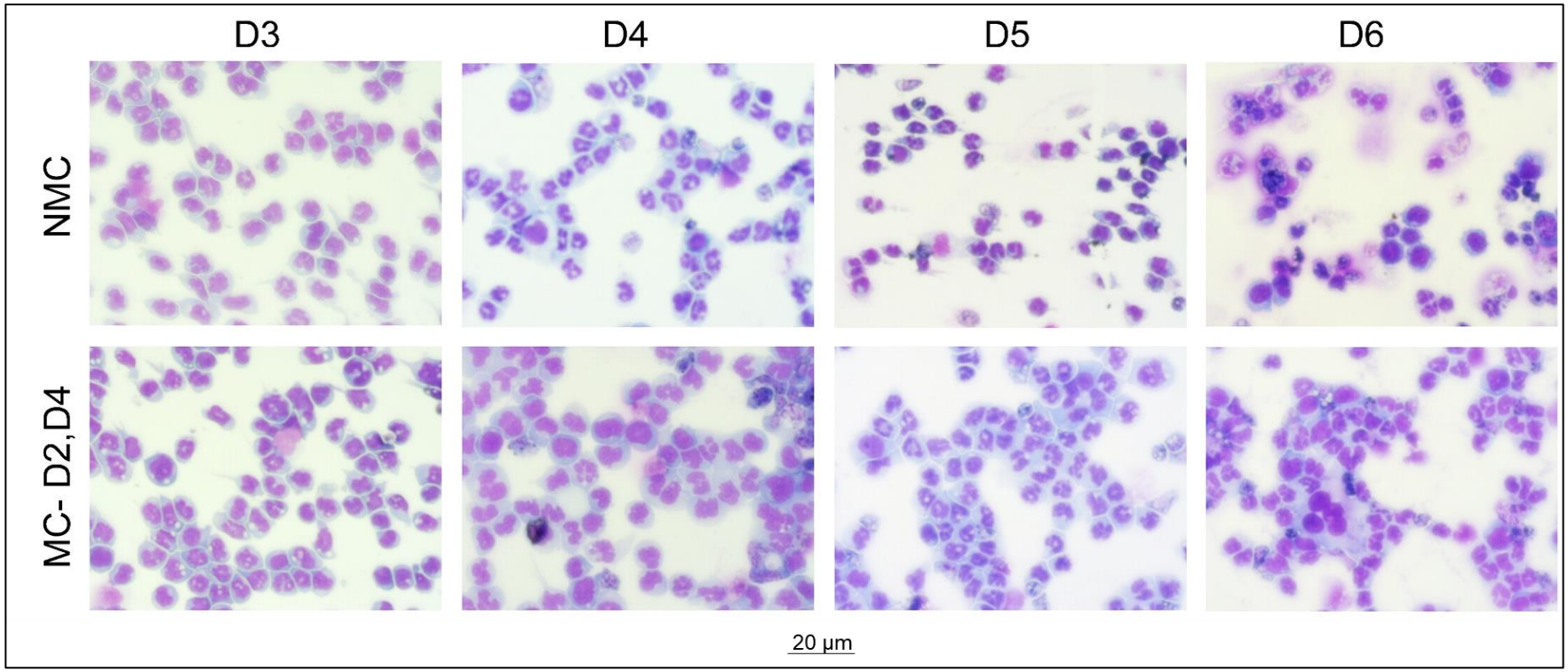


Figure 3.7 Representative cytospin slides images of dPLB-985 cells cultured without and with media changes. PLB-985 cells were cultured in differentiation media for 6 days, without and with media changes on days 2 and 4. Cells samples were removed on days 3-6, cytospin slides prepared, stained with Romanowsky stain and viewed under the light microscope. Cells in upper panel (NMC) were cultured in unchanged differentiation media throughout the 6 days period, while cells in the lower panel (MC- D2, D4) were passaged and re-incubated in fresh differentiation media on days 2 and 4 of culture.

3.3.1.3 Differentiation conditions 3

Another set of experiments was then performed using the improved differentiation conditions described above (changing the medium at days 2 and 4), but supplementing the differentiation media with agents known to promote granulopoiesis and/or delay apoptosis in mature blood neutrophils. Hence, the pro-inflammatory cytokines and granulocyte maturation agents, G-CSF and GM-CSF (Parker et al., 2005, Biethahn et al., 1999, Edwards et al., 1989) were added to the differentiation culture following media changes, to test their effects on differentiation and viability of the differentiated PLB-985 cells.

3.3.1.3.1 Effects of G-CSF and GM-CSF on differentiation and viability of dPLB-985 cells

The cytokines G-CSF or GM-CSF, were added to the differentiating PLB-985 cells at the time of media replacements on days 2 and 4 of culture. The number of differentiated cells was counted manually from representative cytospin slides viewed under a light microscope, while the viability was measured using viaCount assay on the flow cytometer. As shown in Figure 3.8, these two cytokines further increased the number of differentiated cells, and also prolonged their survival period (Figure 3.9). By days 5, 6 and 7, G-CSF significantly ($p \leq 0.05$, n=3) increased the proportion of differentiated cells from 70.5% ± 0.38, 67.8% ± 0.88 and 59.9% ± 2.49 (with media replacements only) to 79.4% ± 1.87, 73.7% ± 1.14 and 65.6% ± 0.79 respectively (Figure 3.8A). GM-CSF also significantly increased ($p \leq 0.05$, n=3) the proportion of differentiated cells to 81.7% ± 1.12, 77.4% ± 1.23 and 68.9% ± 2.56, respectively (Figure 3.8B). Interestingly, more dramatic effects on improvements in cell viability were observed in the presence of these cytokines. For example, cell viabilities were

increased significantly ($p \leq 0.01$, n=3) by G-CSF from 60.0% ± 1.59, 41.5% ± 1.19 and 38.2% ± 0.79 (with media change only) to 81.7% ± 2.38, 71.8% ± 1.02 and 66.8% ± 0.94 at days 5,6 and 7 respectively (Figure 3.9A). GM-CSF also significantly increased viability ($p \leq 0.01$, n=3) to 78.7% ± 1.28, 73.9% ± 2.07 and 68.1% ± 4.50 respectively (Figure 3.9B).

In conclusion, these cytokines significantly enhanced the differentiation of PLB-985 cells as well as greatly extended the survival of the differentiated cells. The relationships between differentiation and cell viability in the presence of G-CSF and GM-CSF are shown in Figure 3.10A and B, respectively. Representative cytospin slides images of cells incubated with G-CSF or GM-CSF are shown in Figure 3.11.

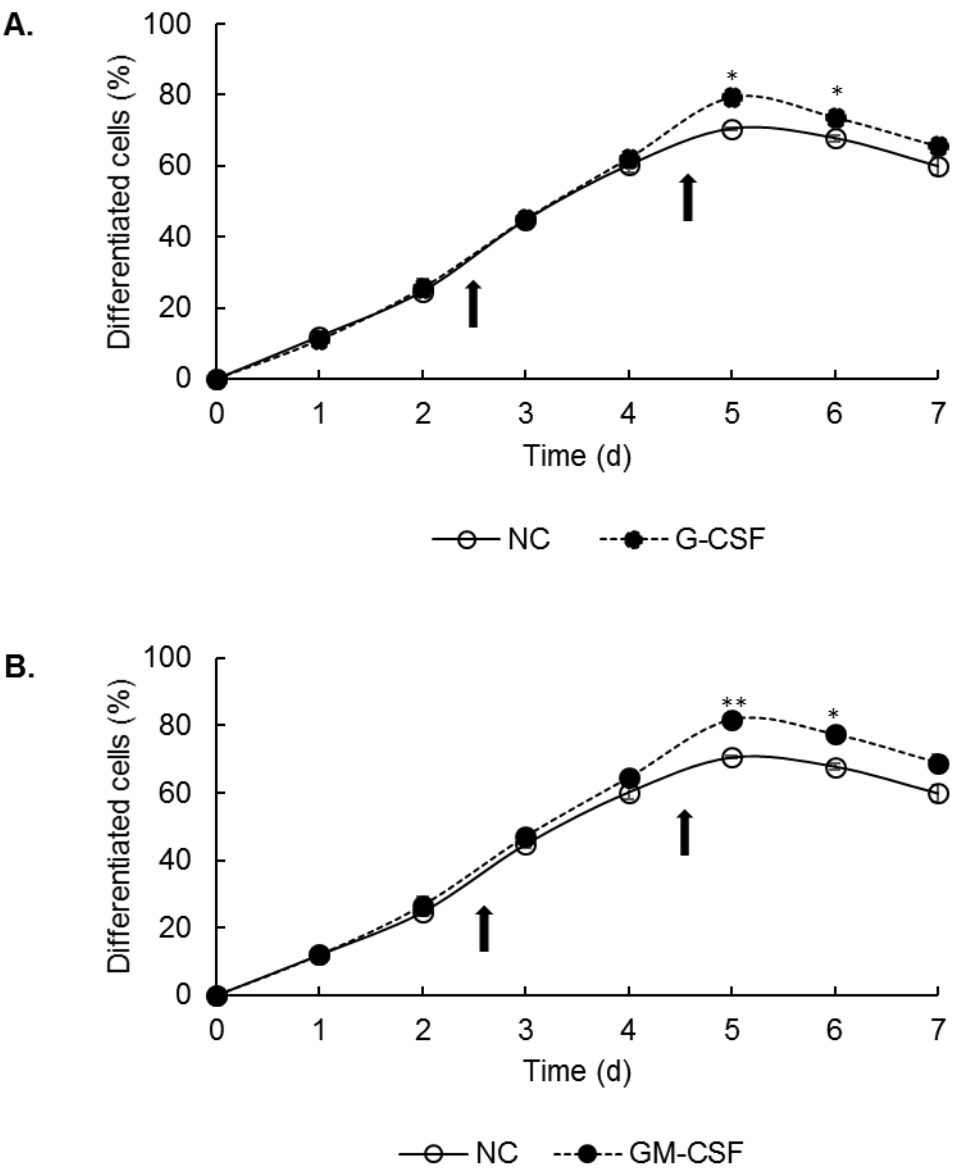


Figure 3.8 Effects of G-CSF and GM-CSF on differentiation of PLB-985 cells. Differentiating PLB-985 cells were incubated without (NC) and with addition of cytokines G-CSF, 10ng/mL (A) or GM-CSF, 5ng/mL (B) after day 2 and 4 following media changes. The number of differentiated cells was counted manually from morphological examination of representative cytospin slides viewed under the light microscope. Arrows indicate times of media changes and cytokine supplementation (after day 2 and 4). Data are expressed as percentages of total cells. (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

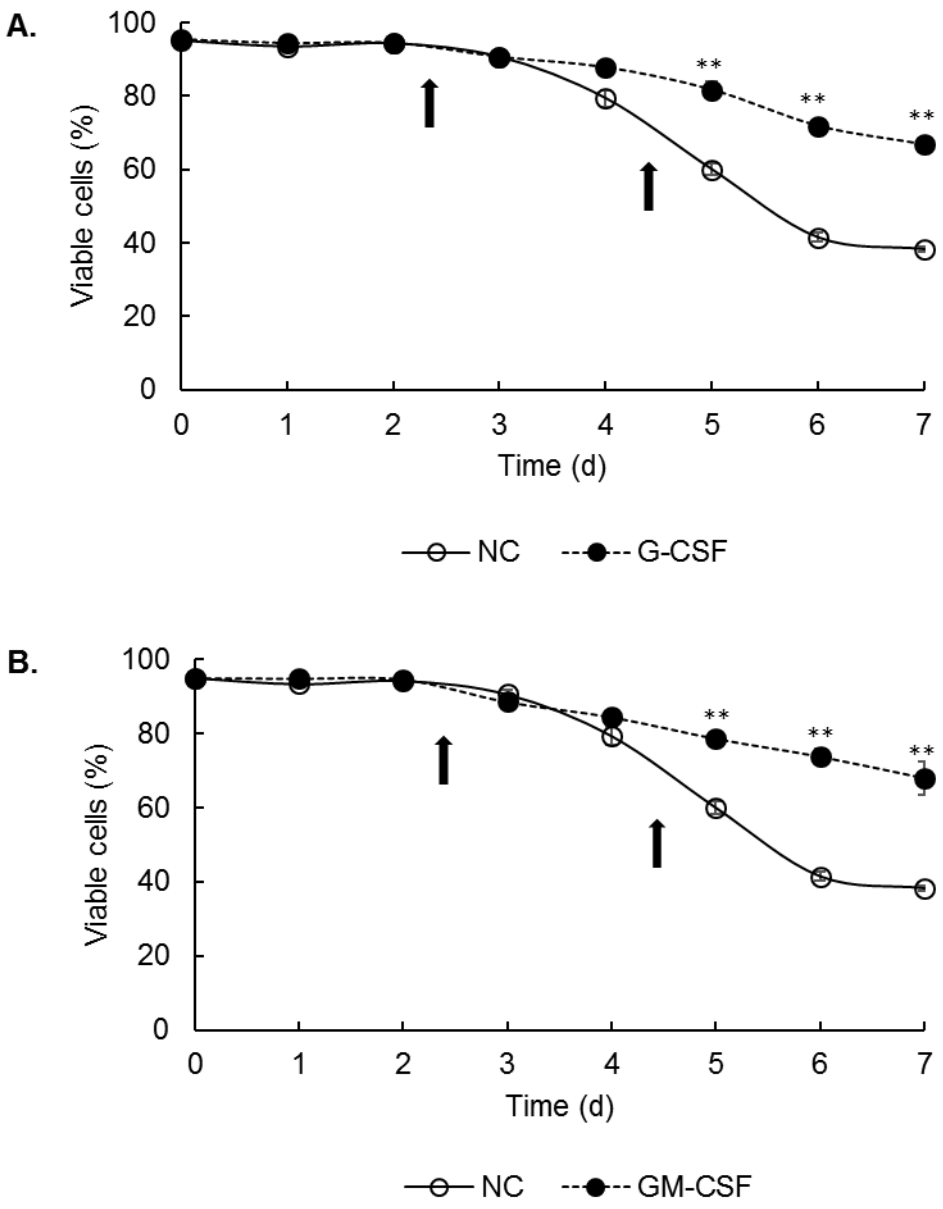


Figure 3.9 Effects of G-CSF and GM-CSF on viability of dPLB-985 cells. Differentiating PLB-985 cells were incubated without (NC) and with addition of cytokines G-CSF, 10ng/mL (A) or GM-CSF, 5ng/mL (B) after day 2 and 4 following media changes. The cells viability was measured using the viaCount assay on the flow cytometer. Arrows indicate times of media changes and cytokine supplementation (after day 2 and 4). Data are expressed as percentages of total cells. (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

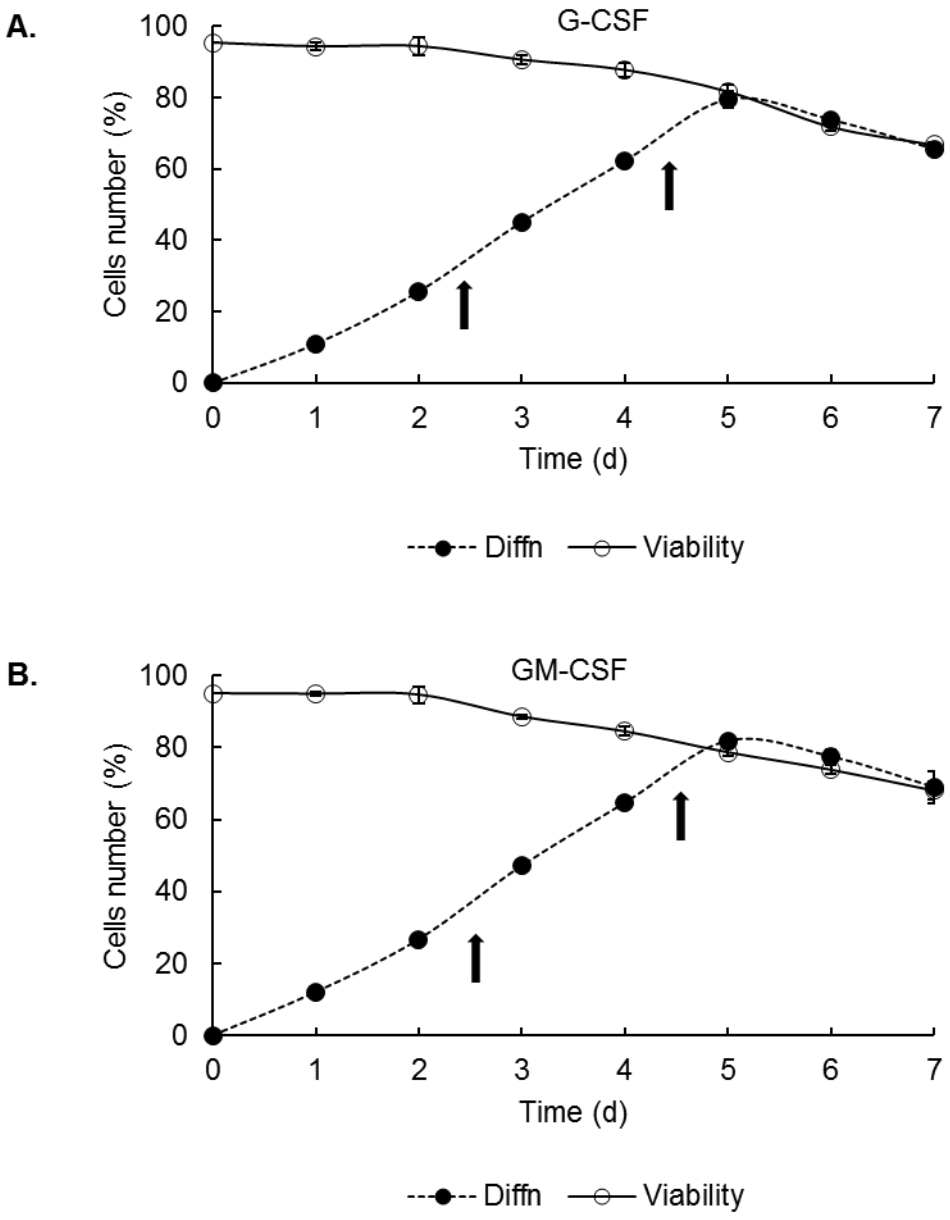


Figure 3.10 Superimposed plots for effects of cytokines on differentiation and viability of dPLB-985 cells. PLB-985 cells were incubated in differentiation media for 7 days, with media changes and addition of cytokines G-CSF, 10ng/mL (A) or GM-CSF, 5ng/mL (B) after day 2 and 4. The number of differentiated cells was counted manually from representative cytospin slides viewed under the light microscope and the cell viability was measured using the viaCount assay on the flow cytometer. Arrows indicate times of media changes and cytokines supplementation (after day 2 and 4). Approximately 80% of the cells are differentiated and viable up to day 7 in both cultures due to apoptosis-delaying effects of the two cytokines. Data are expressed as percentages of total cells.

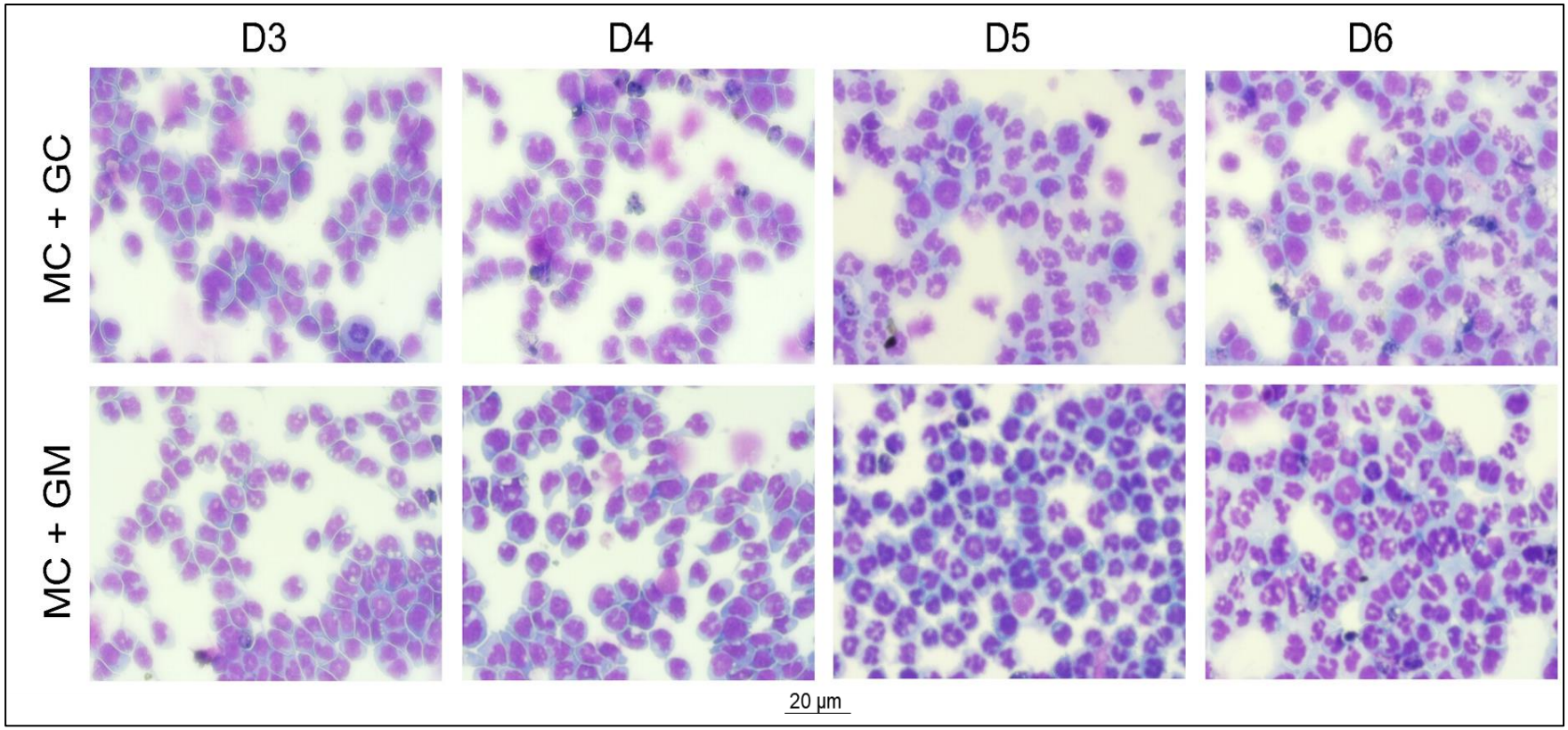


Figure 3.11 Representative cytospin slides images of dPLB-985 cells cultured with media changes and cytokines addition. PLB-985 cells were cultured for 6 days, with differentiation media changes and addition of cytokines G-CSF, 10ng/mL (upper panel) or GM-CSF, 5ng/mL (lower panel) after day 2 and 4. Cells samples were removed on days 3-6, cytospin slides prepared, stained with Romanowsky stain and morphology viewed under the light microscope.

3.3.1.4 Differentiation conditions 4

Another set of experiments was again carried out attempting to further improve the differentiation conditions, by supplementing the differentiation media with Toll-like receptor (TLR) agonists, as some TLR-agonists have also been reported to induce myeloid cells differentiation and maturation, as well as promote neutrophil survival by delaying apoptosis (Kennedy & Deleo, 2009; Parker et al., 2005; Sabroe et al., 2002). The following selected TLR-agonists were therefore added to the differentiation culture during the media changes on days 2 and 4: TLR1/2 agonist Pam3CSK4, TLR2/6 agonist MALP-2, TLR4/10 agonist LPS and TLR7/8 agonist R-848, to determine their effects on differentiation and survival of the differentiated PLB-985 cells. The concentrations used were those reported to optimally delay neutrophil apoptosis.

3.3.1.4.1 Effect of TLR-agonists on differentiation and viability of PLB-985 cells

The TLR-agonists; Pam3CSK4 (200ng/mL), MALP-2 (200ng/mL), LPS (200ng/mL) and R-848 (5µM) were added to the differentiating PLB-985 cells at the time of differentiation media replacements on days 2 and 4 of culture. The number of differentiated cells was counted manually from morphology of representative cytospin slides viewed under a light microscope, while the viability was measured using viaCount assay on the flow cytometer. As shown in Figure 3.12, LPS and R848 significantly ($p \leq 0.05$ or 0.01, n=3) increased the number of differentiated PLB-985 cells, compared to the numbers of differentiated cells obtained after media changes alone on days 3-6, as well as prolonged the survival of differentiated cells by day 6-7 ($p \leq 0.01$, n=3) (Figure 3.13). MALP-2 and Pam3CSK4, on the other hand, showed no

significant effects on differentiation (Figure 3.14) or viability (Figure 3.15) of the differentiated PLB-985 cells.

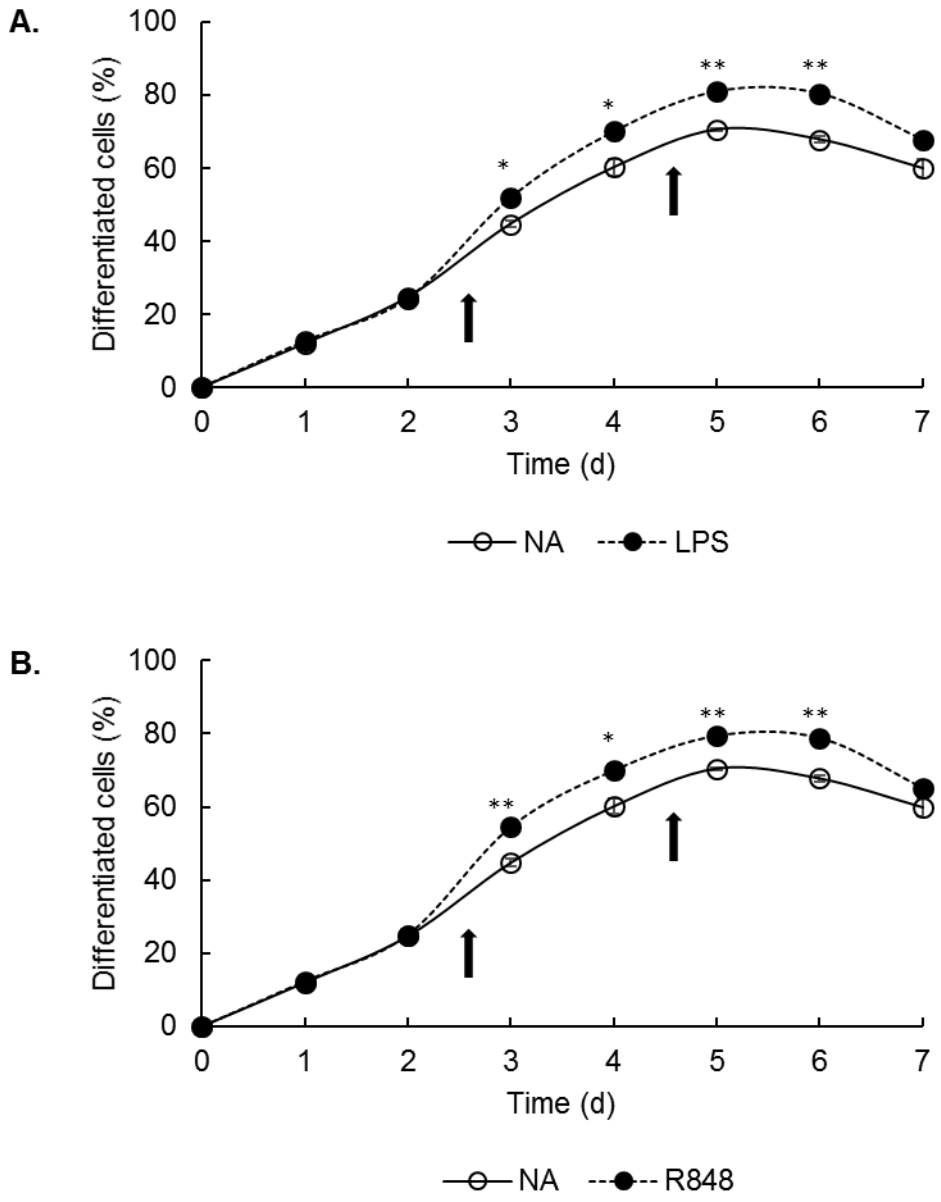


Figure 3.12 Effects of LPS and R848 on differentiation of PLB-985 cells. Differentiating PLB-985 cells were incubated without (NA) and with addition of TLR-agonists LPS, 200ng/mL (A) or R-848, 5µM (B) on days 2 and 4 after media changes. The number of differentiated cells was counted manually from representative cytospin slides viewed under a light microscope. Arrows indicate times of differentiation media changes and LPS or R848 addition. Data are expressed as percentages of total cells. (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

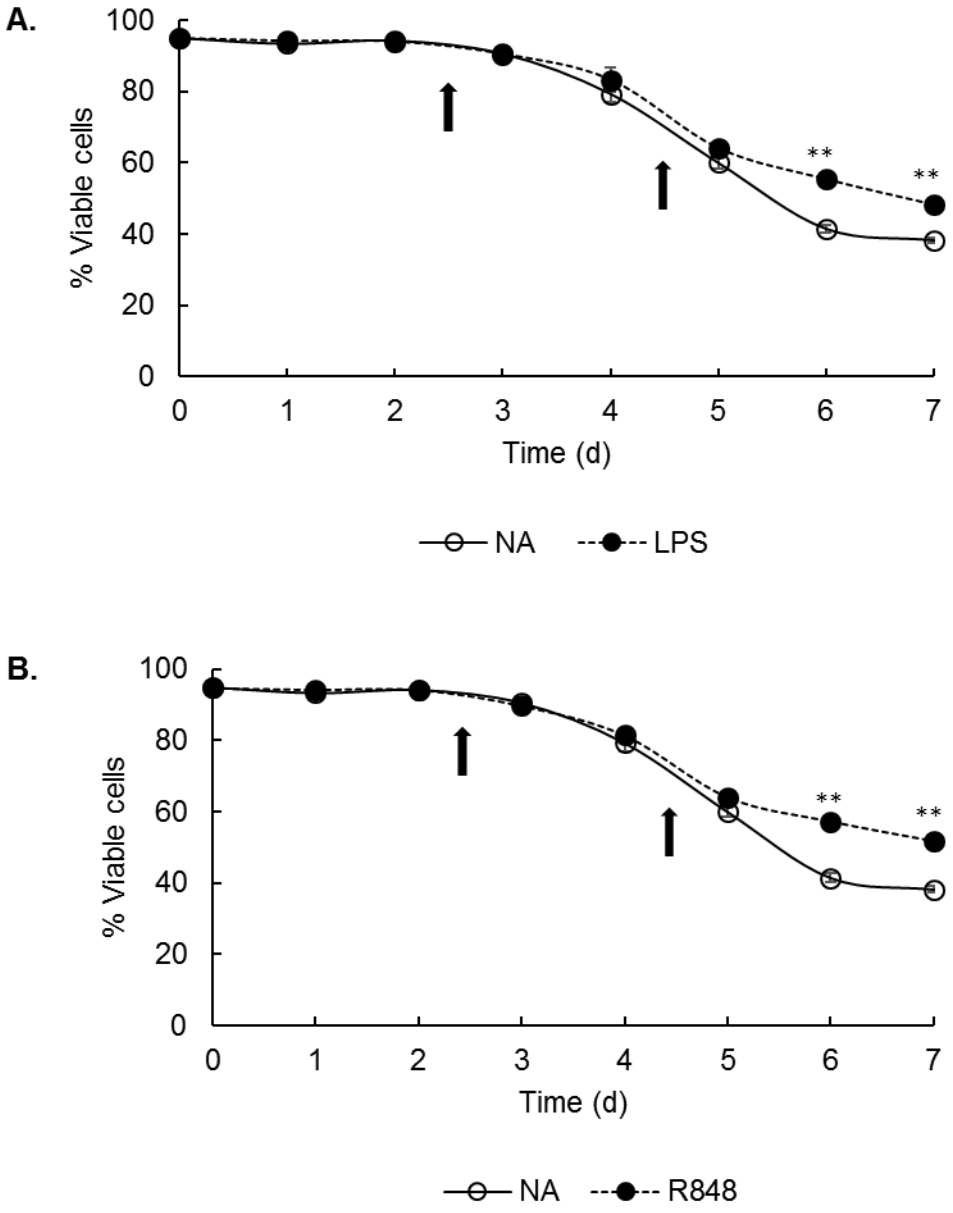


Figure 3.13 Effects of LPS and R848 on viability of dPLB-985 cells. Differentiating PLB-985 cells were incubated without (NA) and with addition of TLR-agonists LPS, 200ng/mL (A) or R-848, 5µM (B) on days 2 and 4 after media changes. The cell viability was measured using the viaCount assay on the flow cytometer. Arrows indicate times of media changes and LPS or R848 addition. Data are expressed as percentages of total cells. (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

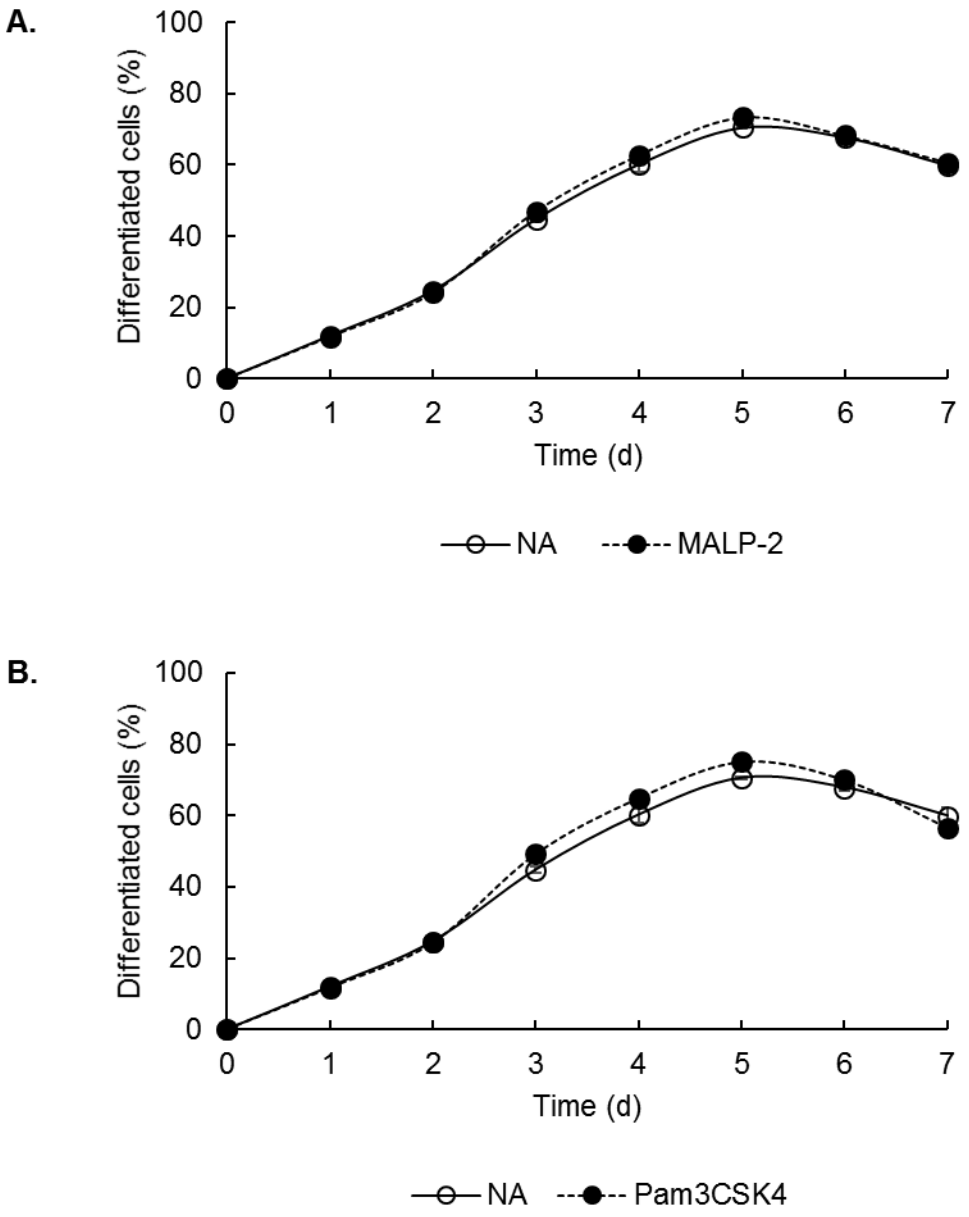


Figure 3.14 Effects of MALP-2 and Pam3CSK4 on differentiation of PLB-985 cells. Differentiating PLB-985 cells were incubated without (NA) and with addition of TLR-agonists MALP-2, 200ng/mL (A) or Pam3CSK4, 5µM (B) on days 2 and 4 after media changes. The number of differentiated cells was counted manually from representative cytospin slides viewed under a light microscope. Arrows indicate times of media changes and MALP-2 or Pam3CSK4 addition. Data are expressed as percentages of total cells. (± SEM, n=3).

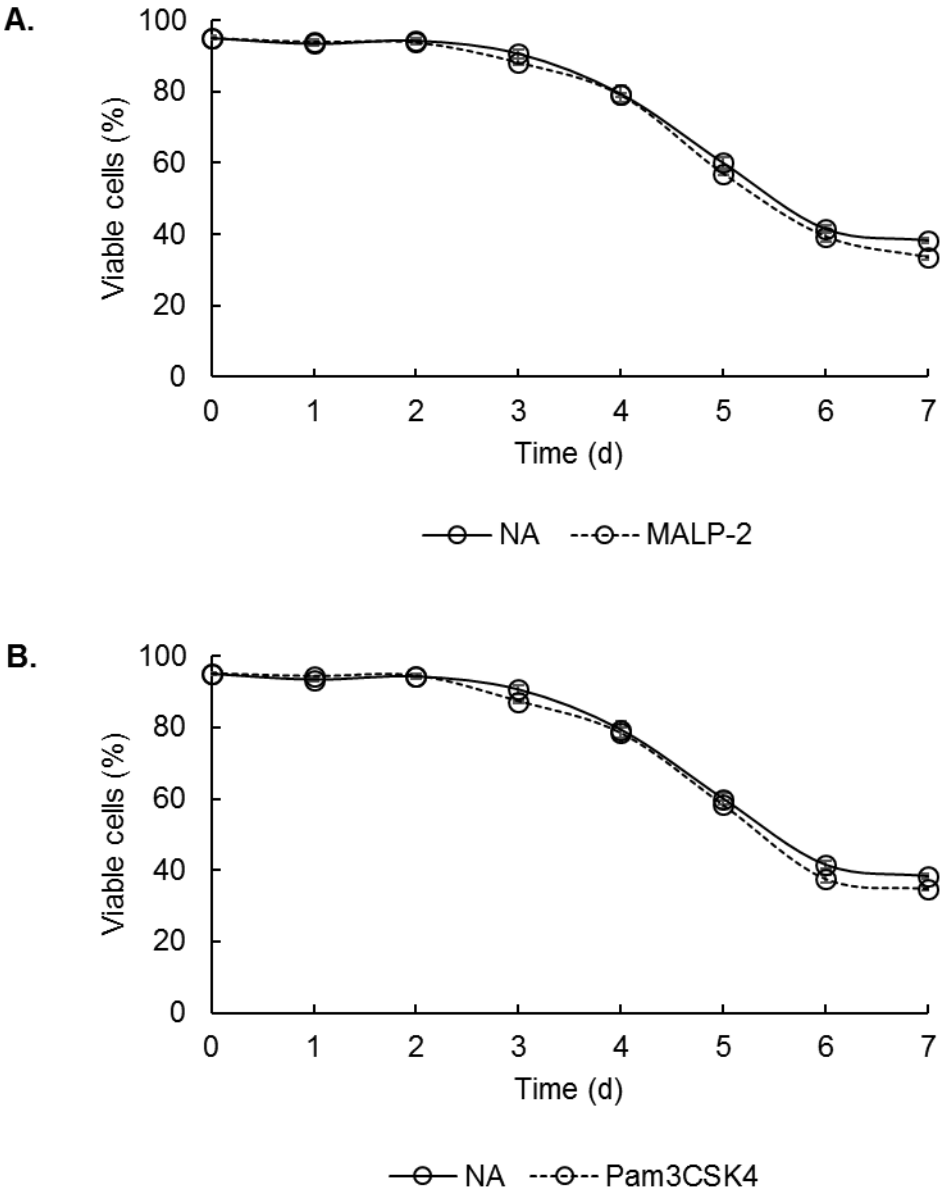


Figure 3.15 Effects of MALP-2 and Pam3CSK4 on viability of dPLB-985 cells. Differentiating PLB-985 cells were incubated without (NA) and with addition of TLR-agonists MALP-2, 200ng/mL (A) or Pam3CSK4, 5µM (B) on days 2 and 4 after media changes. The cell viability was measured using the viaCount assay on the flow cytometer. Arrows indicate times of media changes and MALP-2 or Pam3CSK4 addition. Data are expressed as percentages of total cells.

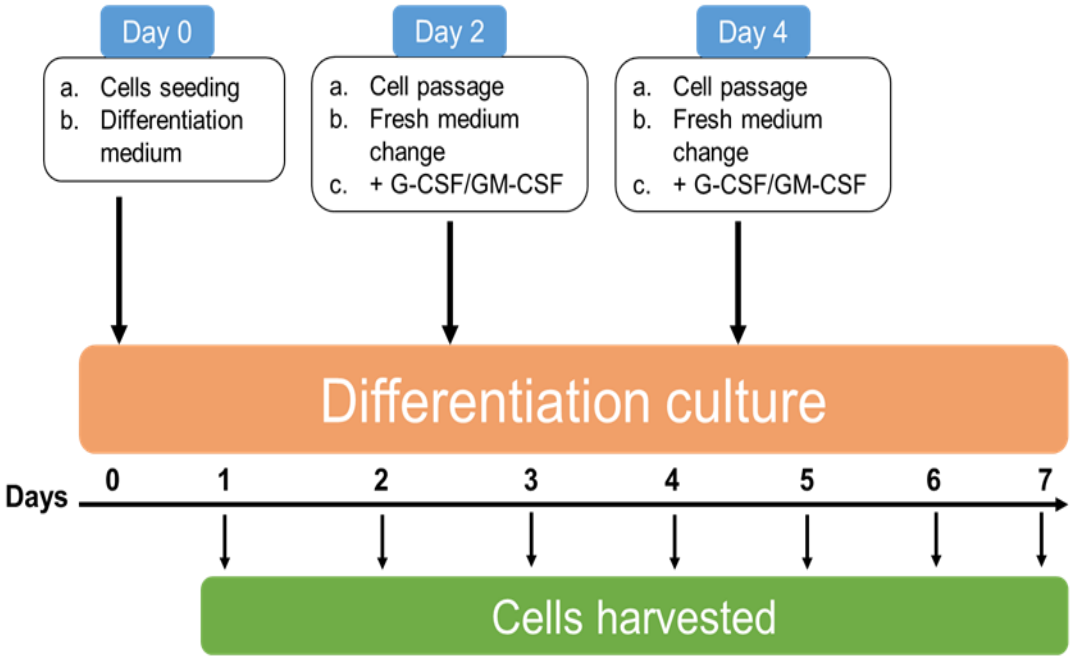


Figure 3.16 Flowchart of procedures for optimisation of differentiation of PLB-985 cells. Exponentially growing PLB-985 cells were initially seeded in differentiation medium. The cells were then passaged, re-suspended in fresh differentiation media, and supplemented with cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) on days 2 and 4 of culture. Each day cells were harvested and re-suspended in fresh differentiation medium, with and without addition of G-CSF or GM-CSF for 30 min, for priming effect before analysis of function. Therefore, up to day 2 in culture, cells will only have been exposed to G-CSF or GM-CSF for 30 min (priming effect). From day 3 onwards, cells were cultured with G-CSF or GM-CSF. This protocol describes the experimental conditions for optimal differentiation efficiency and was used in subsequent experiments in this thesis, henceforth referred to as the "optimised differentiation conditions".

3.4 Discussion and conclusions

The human promyelocytic leukaemia PLB-985 cell line can undergo both granulocyte and monocyte/macrophage differentiation in the presence of appropriate inducing agents. Phenotypically, these are myelomonoblast-like cells, and upon exposure to the appropriate inducing agents, they can be stimulated to differentiate into mature granulocytes or monocytes/macrophages (Shin & Demura, 2012). The aims of this Chapter were to optimise the culture conditions to induce PLB-985 cells to differentiate into mature neutrophil-like granulocytes using ATRA, DMF and sodium pyruvate, in order to establish their usefulness as model of neutrophil differentiation.

The assessment of differentiation in this Chapter was mainly based on morphological properties. Standard morphologic/staining criteria categorizes myeloid cells as immature; comprising blasts, promyelocytes and promonocytes, or as mature; comprising myelocytes, metamyelocytes, bands and segmented neutrophils (Chomienne et al., 1990). The non-differentiated PLB-985 cells are characterised as being at the promyelocytic stage, but the combined effects of ATRA, DMF and sodium pyruvate have successfully transformed them along the myeloid lineage into myelocytes, metamyelocytes, band neutrophils and lastly, segmented neutrophils, the final cells in the myeloid lineage. This pattern of differentiation is analogous to that seen in neutrophil differentiation and maturation during granulopoiesis (see Figure 1.2) (Iwasaki & Akashi, 2007, Lieber et al., 2004).

The differentiated PLB-985 cells exhibited other morphological features of mature neutrophils, including reduced cell size, indented, convoluted and segmented nuclei, decreased number or absence of nucleoli and granulated cytoplasm (Collins et al., 1978). After 3-4 days of induction, metamyelocytes and band neutrophils were the predominant cells in the culture in differentiation medium, whilst after 5-7 days in

culture, segmented neutrophils predominated. In contrast, PLB-985 cells cultured in the absence of differentiation-inducing agents remained in their promyelocytic form, exhibiting large rounded nuclei containing 2-4 nucleoli each, with a dispersed chromatin, agranular cytoplasm and relatively high nuclear/cytoplasmic ratio.

Interestingly, alongside the progressive increase in proportion of cells showing mature neutrophil morphology, there was a parallel increase in the proportion of cells showing morphological features of apoptosis, such as condensation of chromatin and fragmentation of nuclei (Elmore, 2007). Indeed, these apoptotic cells appeared in appreciable numbers only, when or after the differentiated PLB-985 cells with mature neutrophil morphology predominated in the culture, usually after day 5-7 of culture. Apoptosis is a non-pathological, programmed cell death that is constitutively activated as neutrophils age in culture *in vitro* (Edwards et al., 2004). Thus, the differentiated PLB-985 cells have acquired one of the most definitive features of mature neutrophils, namely rapid progression into apoptosis. These observations indicate that differentiated PLB-985 cells may be a good cell line model for the *in vitro* study of neutrophil differentiation and functions, such as the underlying mechanisms of neutrophil apoptosis.

The measurement of PLB-985 cells growth rate following induction of differentiation demonstrated that differentiation significantly decreased the rate of cell growth due to the loss of proliferation capacity caused by ATRA (Degos & Wang, 2001) and DMF (van Dongen et al., 1989). Therefore, as the cells began to differentiate, their proliferation capacity declined and when they became terminally differentiated, they ceased to divide and then underwent spontaneous apoptosis, thus behaving like mature neutrophils. Immunohistochemical studies and electron microscopy have provided evidence that induction of differentiation in leukaemia cell lines is closely associated with inhibition of their proliferation capability (van Dongen et al., 1989).

Therefore, the new differentiation protocol described in this Chapter has proved effective in stimulating PLB-985 cells to differentiate into mature neutrophil-like phenotypes with a typical multi-lobed nucleus and granulated cytoplasm.

The effects of differentiation media changes on days 2 and 4 of culture demonstrated that the proportion of differentiated cells increased significantly as a result of fresh media replacements during the culture period. Most likely, these media changes replenished the nutrient supply to the differentiating cells, maintained the appropriate pH and removed the accumulated metabolic waste products. Media changes also slightly increased the viability of the differentiated cells. There was however, a drastic decrease in the number of viable cells that was evident after day 4 of culture, which is likely attributed to the spontaneous apoptosis within around 24 h of differentiation.

Another significant finding in this study was the importance of cytokines G-CSF and GM-CSF supplementation in promoting both the efficiency of differentiation and extension of viability of the differentiated PLB-985 cells. These observations correlate with previous reported studies on the effects of G-CSF and GM-CSF on proliferation, differentiation and survival of granulocytes and macrophages (Begley et al., 1987). The induction of HL-60 cells to terminally differentiate into mature granulocytes through exposure to the two colony-stimulating factors has been reported (Souza et al., 1986). G-CSF has been demonstrated to induce the formation of neutrophil-like granulocyte colonies in semi-solid agar (Nicola et al., 1983), and to stimulate terminal differentiation, as well as inhibit the self-renewal capacity of murine myelomonocytic leukaemia cells *in vitro* (Burgess & Metcalf, 1980). In a similar way, GM-CSF has also been reported to promote proliferation and function of myelomonocytic cells, increase the number of blood neutrophils by delaying apoptosis and stimulate neutrophil functions, such as reactive oxygen production, by priming (Edwards et al., 1989).

Mature neutrophil undergoes spontaneous apoptosis and are therefore short-lived cells (Edwards, 1994). However, neutrophil apoptosis can be delayed and their lifespan considerably extended by exposure to the cytokines, such as GM-CSF, which acts by increasing their levels of survival protein Mcl-1 (Moulding et al., 1998). Mcl-1 is an anti-apoptotic member of Bcl-2 family proteins with a very high turnover rate, and whose cellular levels correlate with neutrophil survival status (Moulding et al., 1998). It has been demonstrated that GM-CSF increases cellular levels of Mcl-1 by increasing its stability and decreasing its turnover rate that normally occurs via the proteasome (Thomas et al., 2010, Derouet et al., 2004, Edwards et al., 2004).

Selective activation of Toll-like receptors has been reported to promote neutrophil survival by delaying constitutive apoptosis (Parker et al., 2005). Among the four TLRs-agonists added to the differentiation culture in this study, only LPS (TLR-4/10) and R848 (TLR-7/8) significantly increased the number of differentiated cells above those with no agonist (media changes only). These agents also extended the survival of the differentiated cells by day 6-7, but their effects were lower than those of G-CSF and GM-CSF. Ligation of TLR 4 to LPS is described as a principal survival signal in granulocytes (Sabroe et al., 2002).

In contrast, MALP-2 (TLR-2/6) and Pam3CSK4 (TLR-1/2) did not show any significant effects on differentiation or viability of differentiated PLB-985 cells above that obtained with media replacements only. TLRs 2, 4 and 6 have been shown to directly regulate neutrophil apoptosis with TLR 4 being strongly linked to cell survival than TLR 2 or TLR 6 (Kennedy & Deleo, 2009). Recognition of specific molecular patterns in the microbial components (PAMPs) by TLRs is an important mechanism by which the innate immune system senses pathogen invasion, and the subsequent activation of Toll-like receptors induces the expression of specific genes which leads to stimulation of innate immunity (Akira & Takeda, 2004).

The percent differentiation, appearance of the multi-lobed nucleus and granulation of the cytoplasm described in this Chapter, are far better than those reported in publications using similar experimental system (Pivot-Pajot et al., 2010, Ashkenazi & Marks, 2009, Hazan-Eitan et al., 2006, Hiran et al., 2001, Drayson et al., 2001, Martin et al., 1990, Tucker et al., 1987, Souza et al., 1986, Breitman et al., 1980, Collins et al., 1978). Furthermore, apart from demonstrating this high efficiency of PLB-985 cell differentiation into mature neutrophil-like phenotypes, the optimised procedures used in this Chapter have also greatly extended the lifespan of the differentiated PLB-985 cells. To the best of my knowledge, these culture modifications and the resulted enhancement of differentiation and survival of the differentiated cells have not been previously reported.

To this end therefore, the new differentiation protocol described in section 2.3 of this thesis and the optimisation procedures involving media changes and cytokines G-CSF or GM-CSF supplementation on days 2 and 4 described in Figure 3.16, were considered as the optimised differentiation protocol and used in subsequent experiments. Henceforth, referred in this thesis as the “optimised differentiation conditions”.

In conclusion, while previous reports have described the human promyelocytic leukaemia cell line PLB-985 as having the potential to differentiate into neutrophil-like cells under the influence of various chemical agents, the efficiency of differentiation reported was very low. Furthermore, the differentiated cells only partly resembled mature neutrophils in terms of well-defined polymorphic nucleus. The results of the experiments described in this Chapter have confirmed and greatly extended these findings, and have also developed a new differentiation protocol and optimisation procedures to obtain terminally-differentiated PLB-985 cells that mostly resemble mature neutrophils, with prominent segmented, multi-lobular nucleus, granulated

cytoplasm and appreciably extended lifespan. Research in the following Chapters therefore, aimed to define the molecular properties of these differentiated neutrophil-like cells.

Chapter 4: Effect of differentiation of PLB-985 on cell cycle progression and apoptosis

4.1 Introduction

Tissue homeostasis is essential for the proper development of multicellular organisms, and it is maintained through a balance between cell proliferation and cell death. Unregulated cell proliferation, if not compensated for by an appropriate cell death response, may result in pathological conditions, such as neoplasia (Pucci et al., 2000). Apoptosis governs the normal turnover of cells irrespective of their type or tissue location, and therefore, in normal development damaged and unwanted excessive cells are non-pathologically killed and removed through apoptosis (Kennedy & Deleo, 2009).

The cell cycle is a conserved process in multicellular organisms by which cells replicate themselves through a set of cyclic events, comprising two major phases; DNA synthesis (S) and mitosis (M), leading to an accurate duplication of the cell without genetic abnormalities (MacLachlan et al., 1995). Replication of the genome is required for the transmission of genetic information from one cell generation to the next and this occurs during the S (synthesis) phase of the cycle. The genome is then segregated to two new daughter cells during the M (mitosis) phase (see Figure 1.7). There are two preparatory gaps between these two phases; Gap 1 or Growth phase 1 (G1) separates the M phase from the S phase and Gap 2 or growth phase 2 (G2) occurs between the S and M phases. Cells exit the cycle from G1 and enter a dormant state G0 when they undergo differentiation. The orderliness and timing of these events are checked at the G1/S and G2/M boundaries (see Figure 1.8) (Pucci et al., 2000; Yen et al., 1985).

Apoptosis on the other hand, is an evolutionary conserved, essential physiological process of cell death, through which eukaryotes remove defective or unwanted cells, without provoking an inflammatory response, and which occurs during normal development, turnover and certain pathological conditions (Fotedar et al., 1996, Earnshaw, 1995). Indeed, dysregulated apoptosis may lead to disorders, such as cancer and autoimmune diseases (Bursch et al., 2000). As described in section 1.6.2, apoptosis can be triggered through two pathways: extrinsic and/or intrinsic (see Figure 1.5), and the Bcl-2 protein family are the main regulators of the intrinsic apoptotic pathway. The Bcl-2 protein family can be divided into two groups namely, *anti-apoptotic* proteins (e.g. Bcl-2, Bcl-X_L and Mcl-1) and *pro-apoptotic* proteins (e.g. Bak, Bax and Bid), according to their functional contribution in the regulation of apoptosis (see Table 1.1). Mature blood neutrophils express a range of *pro-apoptotic* members of the Bcl-2 family proteins, including Bax and Bak which may partly explain their ability to undergo apoptosis spontaneously and rapidly (Edwards et al., 2004, Moulding et al., 2001).

Several lines of evidence suggest that cell cycle and apoptosis are interconnected to some extent. This is supported by the fact that regulation of cell cycle progression may trigger or prevent programmed cell death, depending on the context of the cell. Tumour suppressor genes, such as cyclin-dependent kinases (Cdks), c-*Myc* and the dominant oncogenes *p53* and RB, are important links between the cell cycle and apoptosis. These genes are involved in proliferative pathways and are not part of cell's apoptotic machinery, but there is strong evidence which indicates that they may trigger progression into apoptosis (Bursch et al., 2000). Cell cycle arrest and/or DNA damage induces apoptosis in tumour cells (reviewed in Evan & Vousden, 2001). Furthermore, there are several morphological characteristics that are common to cells undergoing mitosis and apoptosis, which may indicate a connection between the two processes. Cells become rounded, shrunk, lose substrate attachment, have

condensed chromatin and blebs appear on their membrane during both processes (Nagata, 2000, Monczak et al., 1997).

Although the cell cycle and apoptosis share many morphological features, critical differences exist between them. For example, apoptotic cells exhibit DNA degradation, which gives rise to DNA fragments in multiples of 180bp and cross-linkage of membrane proteins. These processes make the plasma membrane more rigid (Nagata, 2000). In addition, apoptotic cells are subsequently phagocytosed by macrophages or adjacent endo- or epi-thelial cells (Brenner & Mak, 2009). Contrastingly, during mitosis cells exhibit DNA segregation, followed by cytokinesis resulting in cell division into two viable and identical daughter cells (Bursch et al., 2000, Pucci et al., 2000). Hence, there is little or no evidence, to indicate that cell cycle and programmed cell death share common molecular mechanisms (Fotedar et al., 1996). Circulating blood neutrophils are mature, terminally-differentiated, non-dividing cells that have lost the ability to undergo the cell cycle or DNA replication and die constitutively by apoptosis (Edwards, 1994).

As presented in Chapter 3, differentiation of PLB-985 cells significantly decreases their rate of proliferation due to the effects of ATRA and DMF, which inhibit DNA replication and cell proliferation following induction of differentiation (Degos & Wang, 2001; van Dongen et al., 1989). There was also, a progressive increase in the number of cells exhibiting apoptotic morphologies, in parallel with an increase in the number of cells exhibiting mature neutrophil morphology, especially after day 5 of induction, when differentiated cells predominated in the culture. This finding correlated with the observation reported by Degos & Wang that leukaemia cell lines induced to differentiate terminally to granulocytes in response to ATRA, subsequently underwent spontaneous apoptosis (Degos & Wang, 2001).

It was therefore necessary to determine the rate of apoptosis of terminally-differentiated PLB-985 cells and isolated blood neutrophils, as mature neutrophils have a very short half-life due to their ability to undergo spontaneous apoptosis (Edwards, 1994). Taken altogether, these data suggest a connection between differentiation, growth arrest and apoptosis which will be investigated further in this Chapter.

The aims of the studies described in this Chapter are therefore to:

1. Measure cell cycle parameters in differentiation-induced and non-induced PLB-985 cells and mature blood neutrophils.
2. Determine the effects of differentiation of PLB-985 cells on apoptosis.
3. Investigate the relationship between cell cycle progression and programmed cell death in differentiated PLB-985 cells.
4. Determine whether differentiated PLB-985 cells resemble isolated blood neutrophils in their cell cycle parameters and expression of proteins that regulate apoptosis.

4.2 Methods

All methods used in this Chapter are described in detail in Chapter 2. Cell cycle progression was analysed using the flow cytometer. For analysis of cell cycle parameters, cells were fixed with ethanol, and then stained with propidium iodide, which binds DNA and produces fluorescence that is proportional to the DNA concentration per cell, and which varies as the cell progresses through the cycle. The assay reveals the distribution of viable cells in three major phases of the cell cycle:

G1, S and G2. It also allows detection of apoptotic cells in the quiescent phase G0, with a decreased DNA content. Flow cytometry was used to analyse apoptosis.

Expression of the Bcl-2 family of apoptotic proteins was determined by western blot analysis, performed with a range of human anti-Bcl-2 protein family antibodies. This approach was used to detect the expression levels of *anti-apoptotic* members of the Bcl-2 protein family, such as Mcl-1, Bcl-X_L and Bcl-2; as well as *pro-apoptotic* members, such as Bax and Bak. Morphological examination of apoptosis in differentiated PLB-985 cells and isolated blood neutrophil was performed under a light microscope, after preparation of cytospin slides and Romanowsky staining.

4.3 Results

4.3.1 Effects of differentiation of PLB-985 cells on cell cycle kinetics

PLB-985 cells were cultured in the absence (PLB-985) and presence (dPLB-985) of differentiation-inducing agents, with unchanged media for 6-days period. The cells were prepared, stained with propidium iodide and cell cycle kinetics were analysed on days 1, 3, 5 and 6 using the flow cytometer. As shown in Figure 4.1, on day 1 most of the cells were found accumulated in G1 (PLB: 43.81% ± 3.38%, dPLB: 49.73% ± 4.14%), followed by G2 (PLB: 27.97% ± 8.48%, dPLB: 26.75% ± 1.30%) phases. Differentiation of PLB-985 cells induced cell cycle arrest after 3 days of culture, as indicated by increases ($p \leq 0.05$, n=3) in the number of cells in G0, suggesting G0 arrest/apoptosis.

In parallel, the numbers of cells in S phase progressively decreased after day 3-6 of culture, whereas the percentage of cells in G2 phase increased, suggesting blockage of DNA replication and inhibition of normal cell cycle progression. These changes paralleled those reported in Chapter 3 and are indicative of cells in differentiation medium first undergoing cell cycle arrest as they mature into neutrophils that lack proliferative activity, and then subsequently undergoing apoptosis. Representative flow cytometer traces are shown in Figures 4.3 and 4.4. Figure 4.3 shows dot plots of DNA staining from which the cell cycle parameters (Figure 4.4) were calculated.

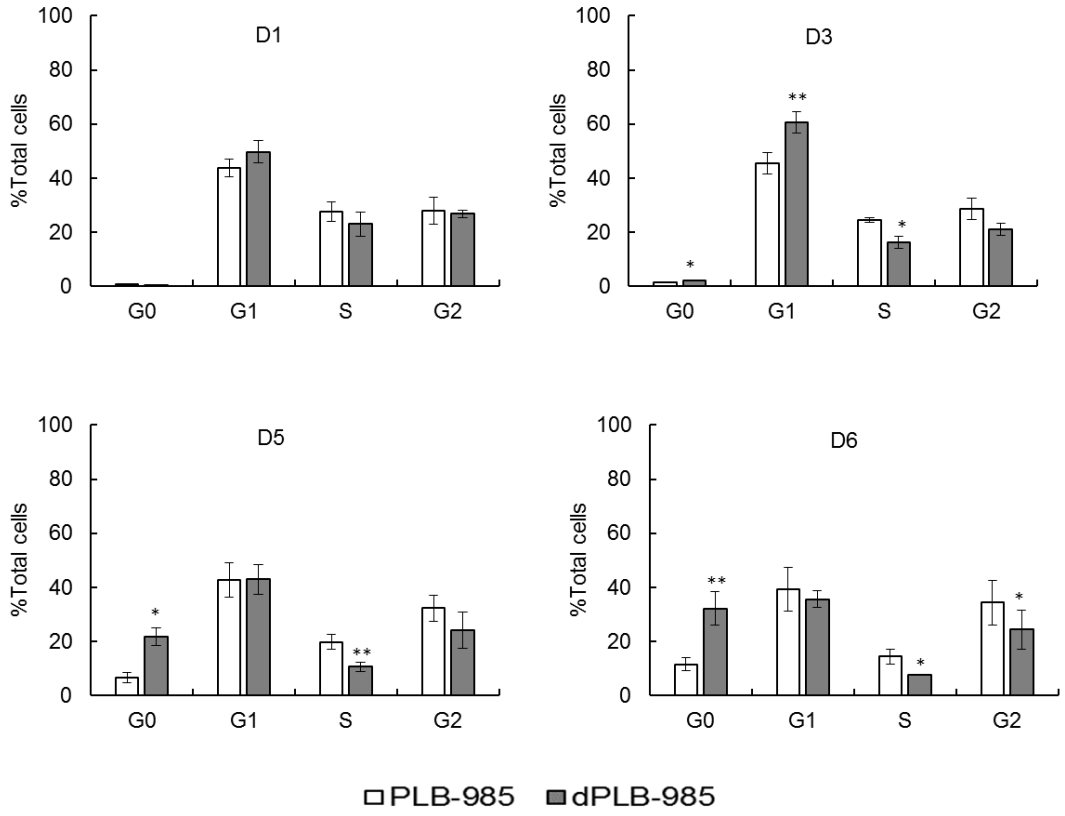


Figure 4.1 Effect of differentiation of PLB-985 cells on cell cycle parameters. Differentiation-induced (dPLB-985) and non-induced (PLB-985) cells were cultured for 6 days with unchanged culture media. Cells were prepared, stained with propidium iodide and cell cycle kinetics were analysed on days 1, 3, 5 and 6 using the flow cytometer. After days 3-6 of culture, there was significant, progressive increase in number of cells that accumulated in G0 and decreased number of cells in S and G2 phases in dPLB-985. Data are expressed as percentages of total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

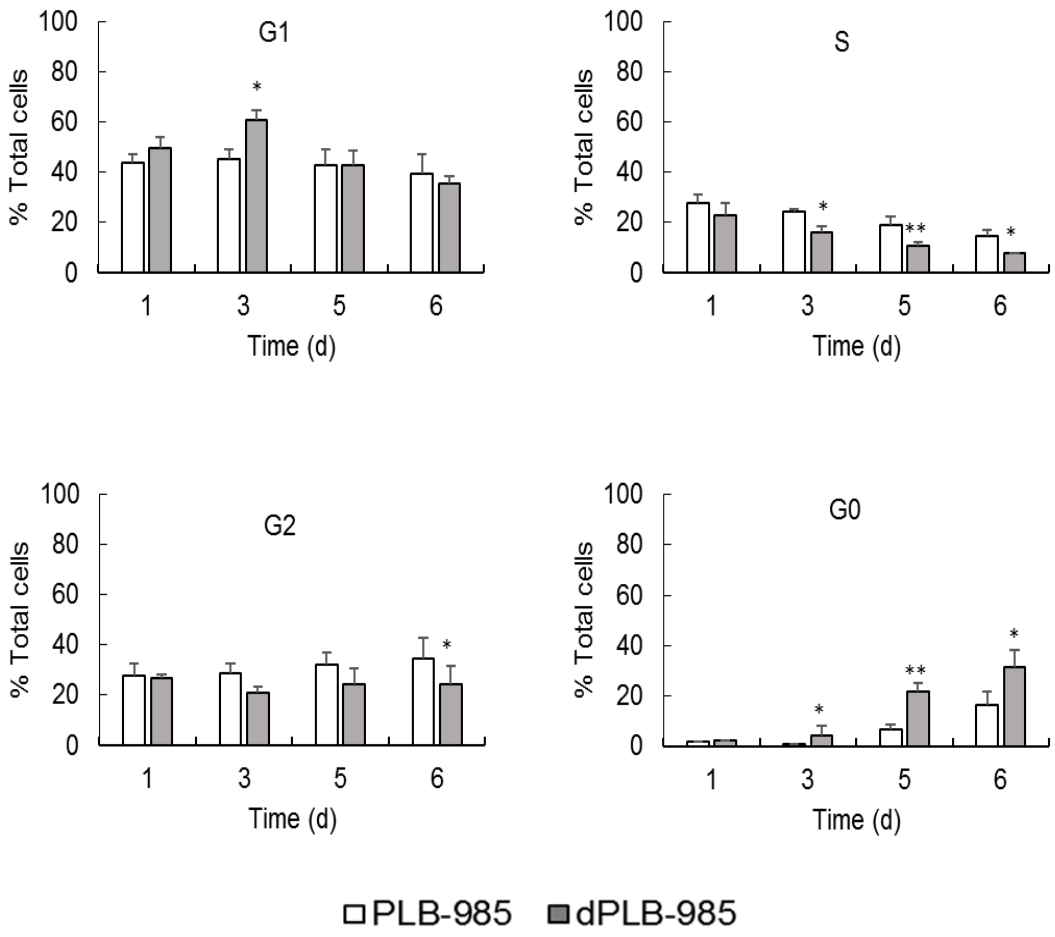


Figure 4.2 Proportions of PLB-985 and dPLB-985 cells in the different phases of cell cycle. Differentiation-induced (dPLB-985) and non-induced (PLB-985) cells were cultured for 6 d with unchanged culture media. Cells were prepared, PI stained and cell cycle parameters were analysed on days 1, 3, 5 and 6 using the flow cytometer. Most of the cells were in G1 phase but after days 3-6 of culture there was a significant increase in the number of cells in G0 and decrease in the number of cells in S and G2 phases. These cell cycle parameters were calculated from the DNA distribution profiles shown in Figure 4.3. Data are expressed as percentages of total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

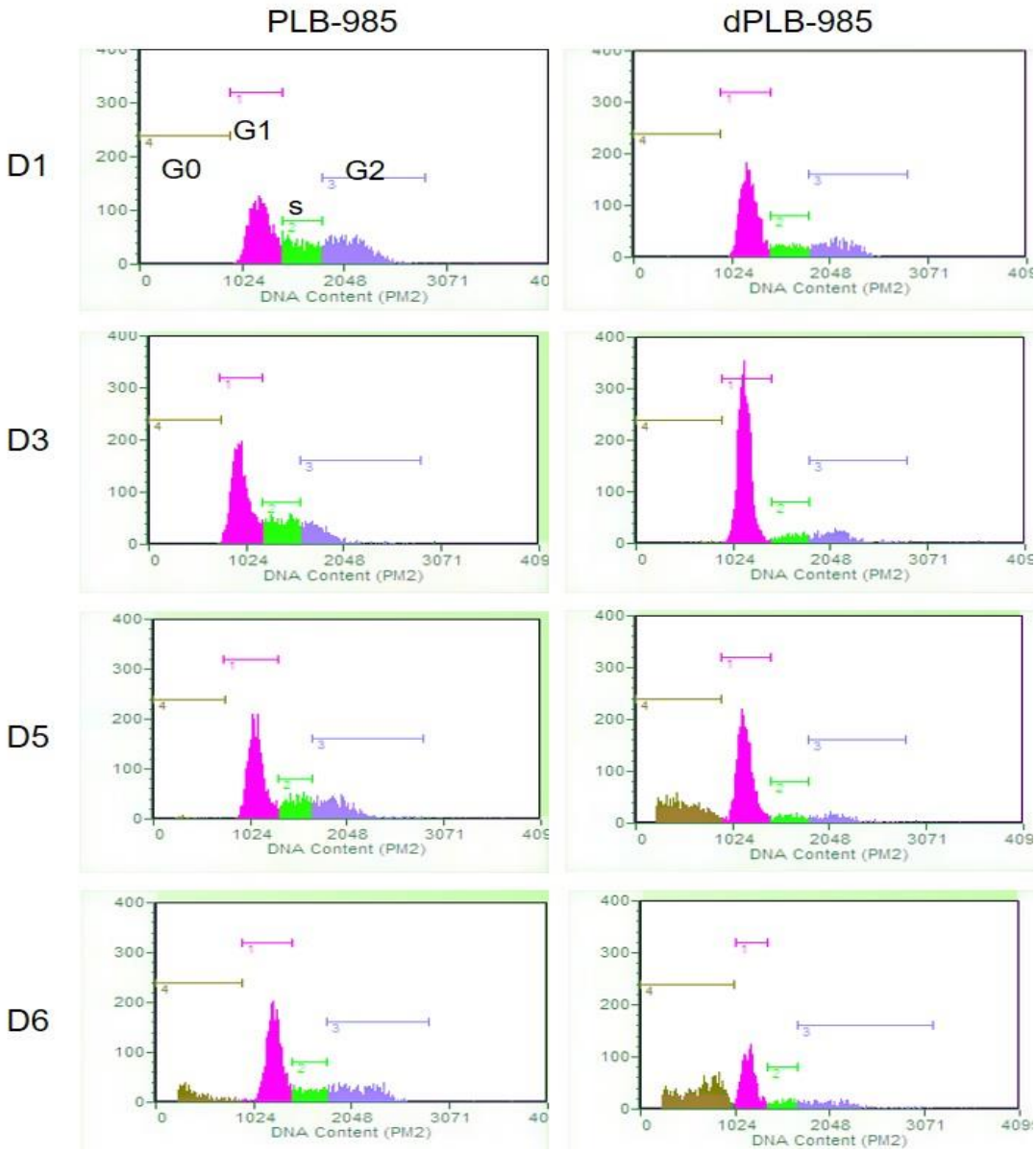


Figure 4.3 Representative flow cytometer histogram traces for cell cycle progression of PLB-985 and dPLB-985 cells. Cells were cultured for 6 d with unchanged culture media. Cells were prepared, stained with propidium iodide and cell cycle kinetics were analysed on days 1, 3, 5 and 6 using the flow cytometer. After days 3-6 of culture there was increased accumulation of cells in G0 and decreased number of cells in S and G2 phases, which were higher in dPLB-985 cells.

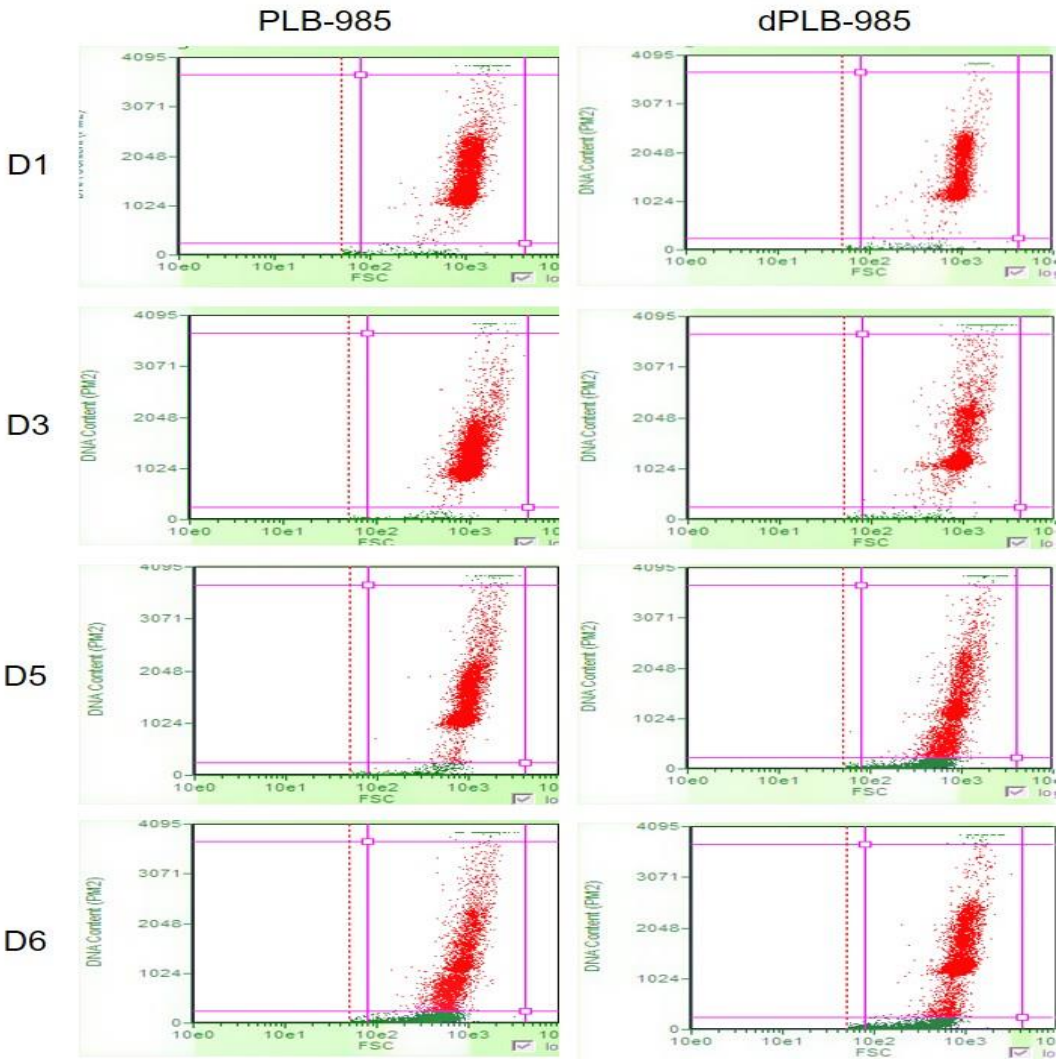


Figure 4.4 Representative flow cytometer dot plots showing DNA contents of PLB-985 and dPLB-985 cells. Cells were cultured for 6 d with unchanged culture media. Cells were prepared, stained with propidium iodide and cell cycle parameters were analysed on days 1, 3, 5 and 6 using the flow cytometer. After days 3-6 of culture there was accumulation of cells with low DNA contents (lower, green dots) indicating cells in G0 (apoptosis) and numbers of these cells were higher in dPLB-985 cells.

4.3.2 Effect of media changes on cell cycle kinetics of dPLB-985 cells

The results in Chapter 3 (Figure 3.16) showed that changing the culture media after days 2 and 4 and supplementing the differentiation media with G-CSF and GM-CSF improved the efficiency of differentiation, and enhanced the survival of the differentiated cells. It was then necessary to determine if these morphological features were accompanied by changes in the cell cycle kinetics of these cells. Cells were cultured in differentiation media for 6 days, without (NMC) and with media changes on day 2 and 4 (MC). Cells were prepared, stained with propidium iodide and cell cycle parameters analysed on days 5 and 6 using the flow cytometer.

As shown in Figure 4.5, in culture with media changes (MC) there was decrease in the number of cells in G0 and G2 phases, but only G0 reached statistically-significant ($p \leq 0.05$) levels. In contrast, there were more cells in G1 and S phases in culture with differentiation media changes, but only G1 reached significant ($p \leq 0.01$) levels. These results indicate that media changes decreased the accumulation of cells in G0 and G2 (apoptosis) and increased those in G1 and S (enhanced differentiation and survival), as the cells aged in culture. Representative flow cytometer traces for cells cycle stages histogram and corresponding dot plots of DNA staining are shown in Figures 4.6.

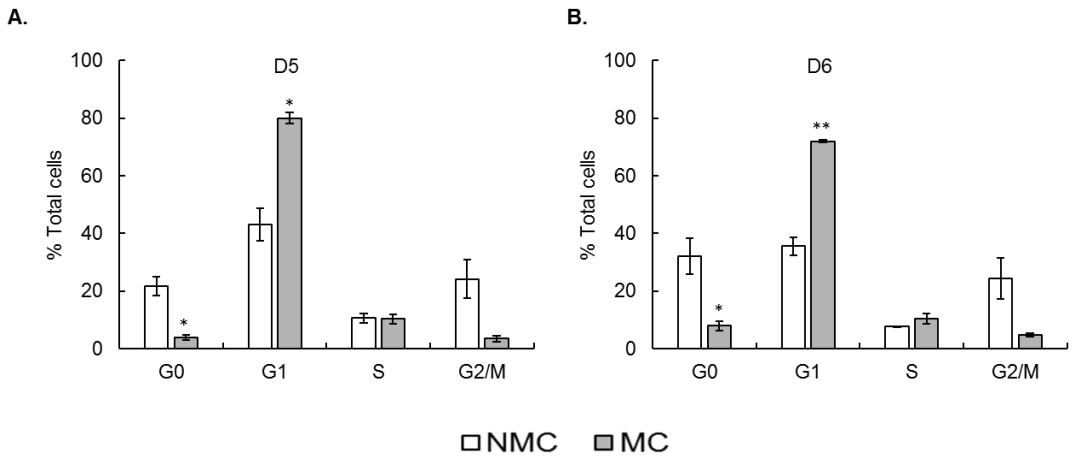


Figure 4.5 Effect of differentiation media changes on cell cycle kinetics of dPLB-985 cells. Differentiating PLB-985 cells were cultured for 6 d without (NMC) and with (MC) differentiation media changes on days 2 and 4. Cells were prepared, stained with propidium iodide and cell cycle parameters were analysed on days 5 and 6 using the flow cytometer. Cells cultured with media changes (MC) had fewer number of cells in G0 and G2 phases, as well as increased number of cells in G1 phase. These cell cycle parameters were calculated from the DNA distribution profiles shown in Figure 4.6A. Data are expressed as percentages of total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

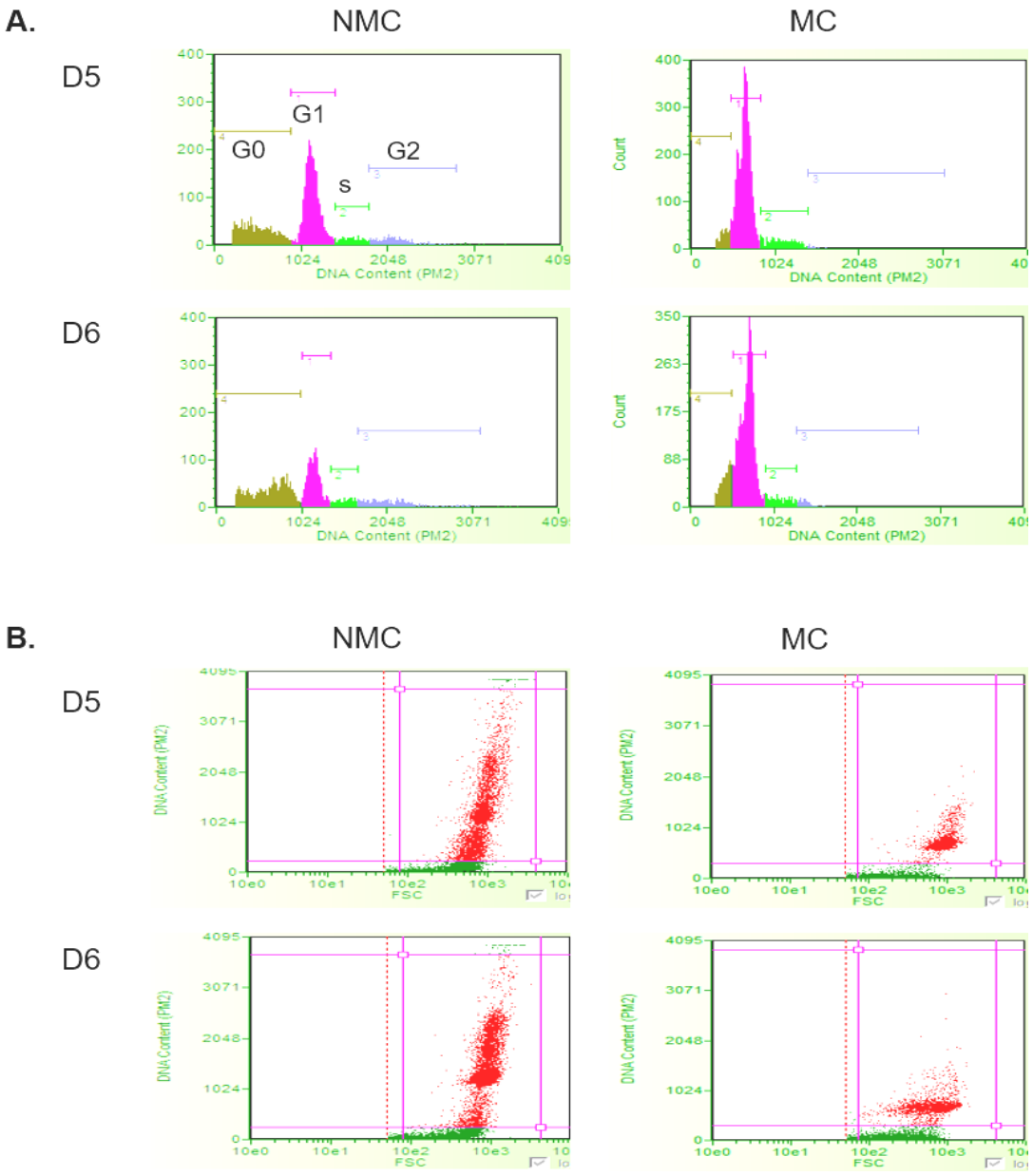


Figure 4.6 Representative flow cytometer traces of cell cycle parameters of dPLB-985 cells cultured without and with media changes. Differentiating PLB-985 cells cultured for 6 d without (NMC) and with (MC) media changes were stained with propidium iodide and cell cycle parameters were analysed on days 5 and 6 using the flow cytometer. Cells cultured with media changes (MC) had fewer number of cells with low DNA contents (G0 phase, lower dots). (A) Histogram plot of DNA distribution profiles (B) Dots plots of DNA contents.

4.3.3 Effect of G-CSF and GM-CSF on cell cycle parameters of dPLB-985 cells

Differentiating PLB-985 cells cultured for 6 days were incubated without (NC) and with cytokines, G-CSF (10ng/mL) or GM-CSF (5ng/mL) after media changes on days 2 and 4. The cells were prepared, stained with propidium iodide and cell cycle parameters were analysed on days 1, 3, 5 and 6 using the flow cytometer. Figure 4.7 shows the cell cycle parameters under these conditions. Most of the cells were accumulated in G1 by day 1 (NC: 67.92% ± 3.21%, GC: 63.80% ± 0.22% and GM: 74.34 ± 0.61%). After days 3-6, there was significant increase ($p \leq 0.05$, n=3) in the cells in G0.

Incubation of dPLB-985 cells with G-CSF or GM-CSF during the differentiation media changes on days 2 and 4 of culture increased the number of cells that accumulated in G1 phase, as well as decreases cells in S and G2 phases. These differences were significant ($p \leq 0.05$ or 0.01) from day 3 onwards (Figures 4.7 and 4.8). This suggests that both G-CSF and GM-CSF delay cell cycle arrest and progression into apoptosis, as well as considerably prolong the survival of differentiated cells. Representative flow cytometer traces of dot blots for DNA staining and corresponding traces of histogram for cell cycle stages are shown in Figures 4.9 and 4.10, respectively.

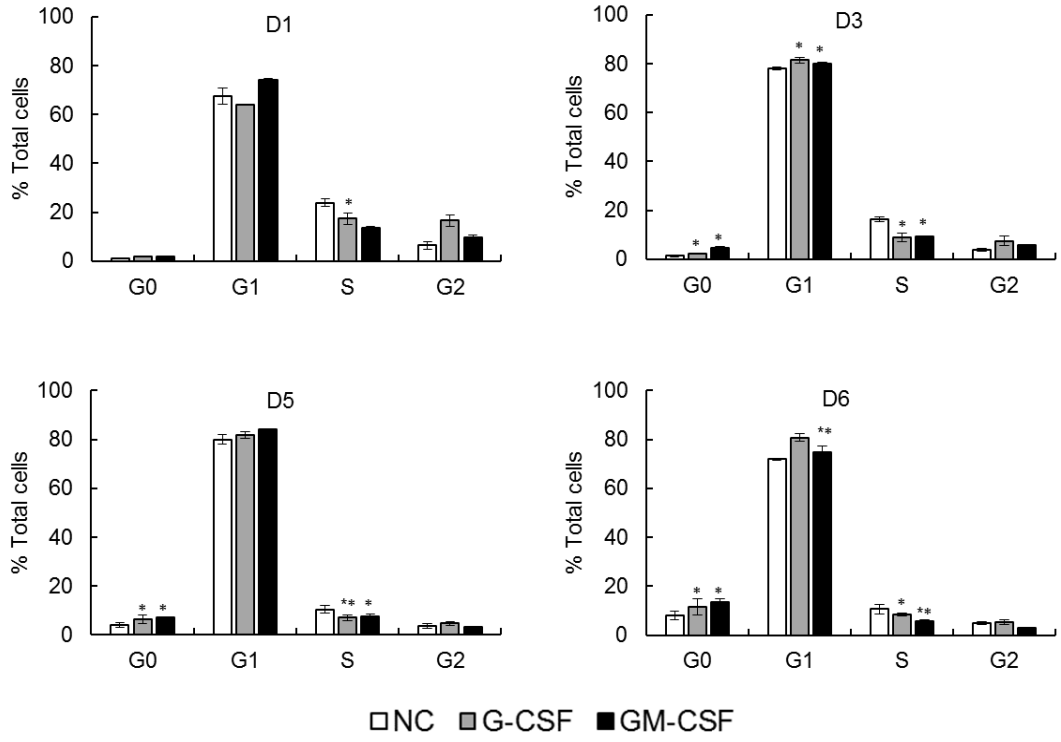


Figure 4.7 Effect of G-CSF and GM-CSF on cell cycle parameters of dPLB-985 cells. Differentiating PLB-985 cells were incubated for up to 6 days in the absence (NC) and presence of cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) after differentiation media changes on days 2 and 4. Cells were stained with propidium iodide and cell cycle parameters were analysed on days 1, 3, 5 and 6 using the flow cytometer. After day 3-6 of culture, there were significant increases in the number of cells accumulated in G0 phase and a decrease in the number of cells in S phase. These cell cycle parameters were calculated from the DNA distribution profiles shown in Figure 4.9. Data are expressed as percentages of total cells (± SEM, n=3), * = $p \leq 0.05$, ** = $p \leq 0.01$ (paired, two-tailed student's t-test).

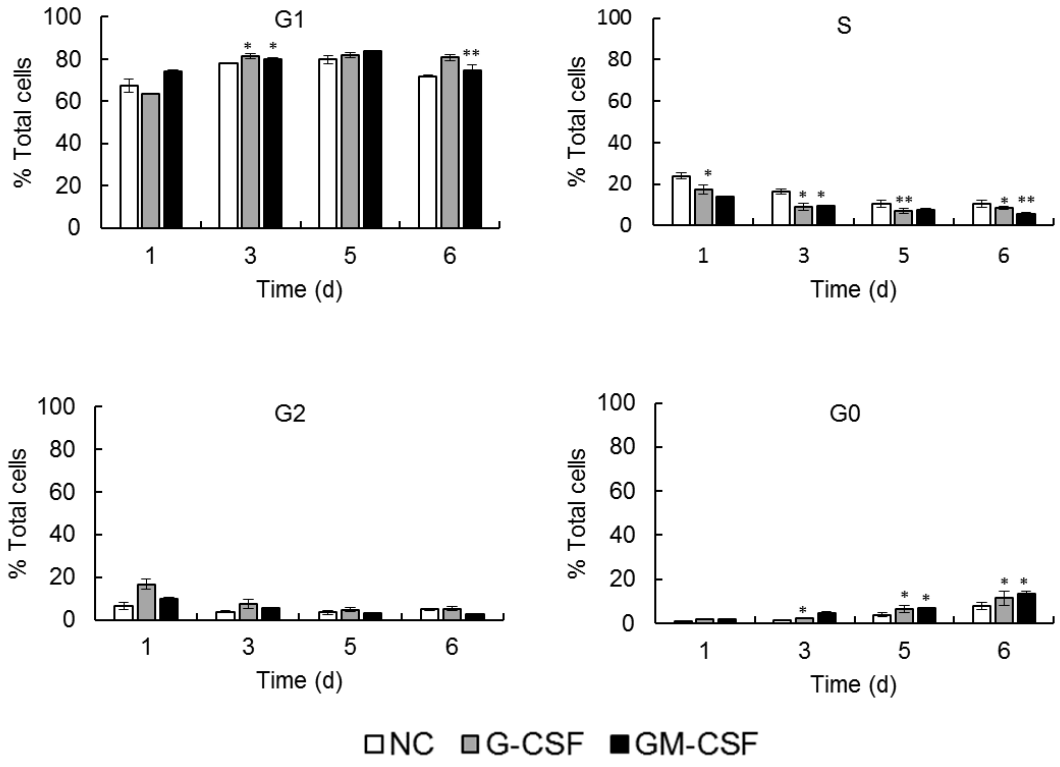


Figure 4.8 Proportions of dPLB-985 cells in the cell cycle phases after cytokine supplementation. Differentiating PLB-985 cells were incubated for 6 days in the absence (NC) and presence of cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) after media changes on days 2 and 4. Cells were stained with propidium iodide and cell cycle kinetics were analysed on days 1, 3, 5 and 6 using the flow cytometer. Most of the cells were initially in G1 phase but after days 3-6 of culture, there was a significant increase in number of cells in G0 phase and decrease in S and G2 phases. These cell cycle parameters were calculated from the DNA distribution profiles shown in Figure 4.9. Data are expressed as percentages of total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

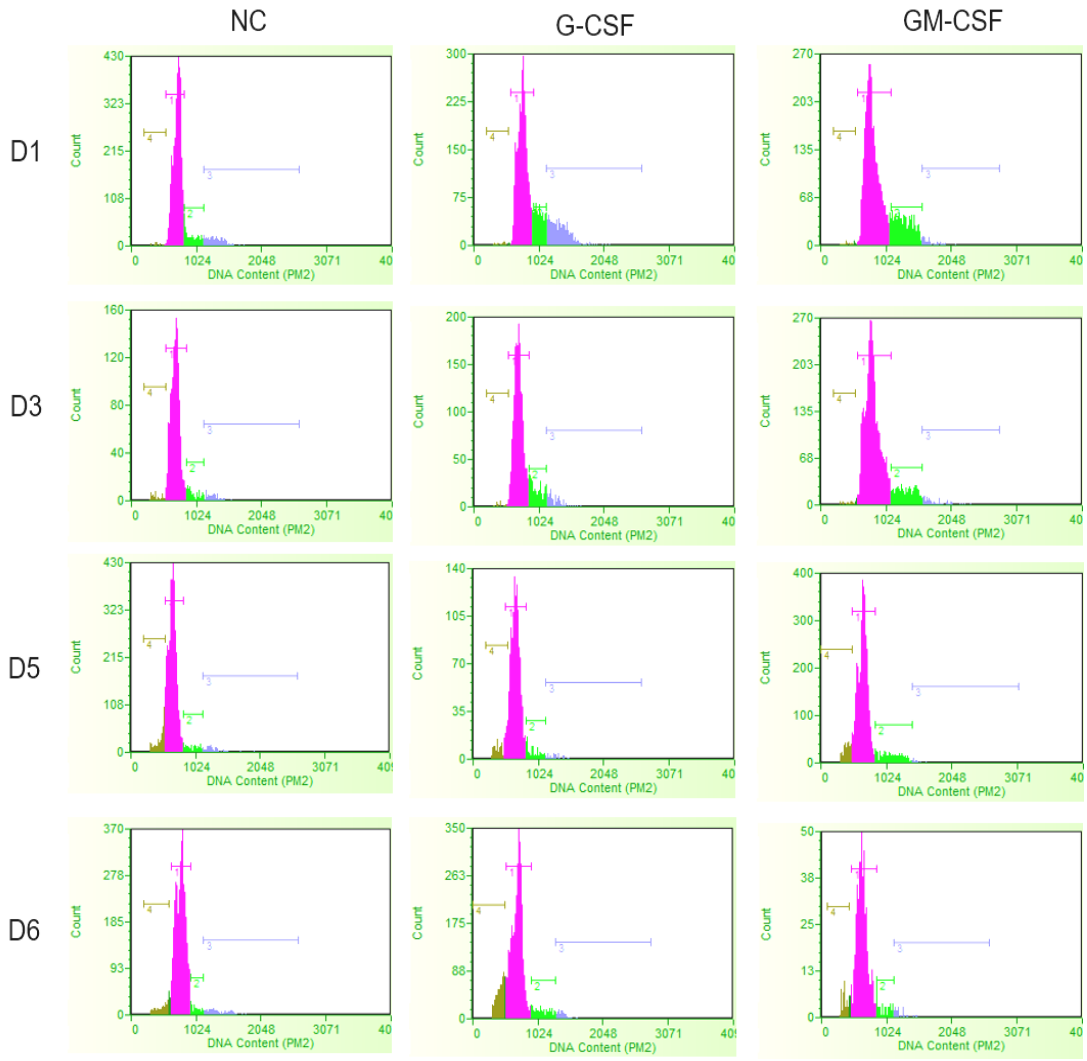


Figure 4.9 Representative flow cytometer histogram plots of cell cycle parameters of dPLB-985 cells cultured with cytokine supplementation. dPLB-985 cells were incubated for 6 days in the absence (NC) and presence of cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) after media changes on days 2 and 4. Cells were stained with propidium iodide and cell cycle parameters were analysed on days 1, 3, 5 and 6 using the flow cytometer. Most of the cells were initially in G1 phase but after days 3-6 of culture, there was an increase of cells in G0 phase (low DNA contents) and decrease in S and G2 phases.

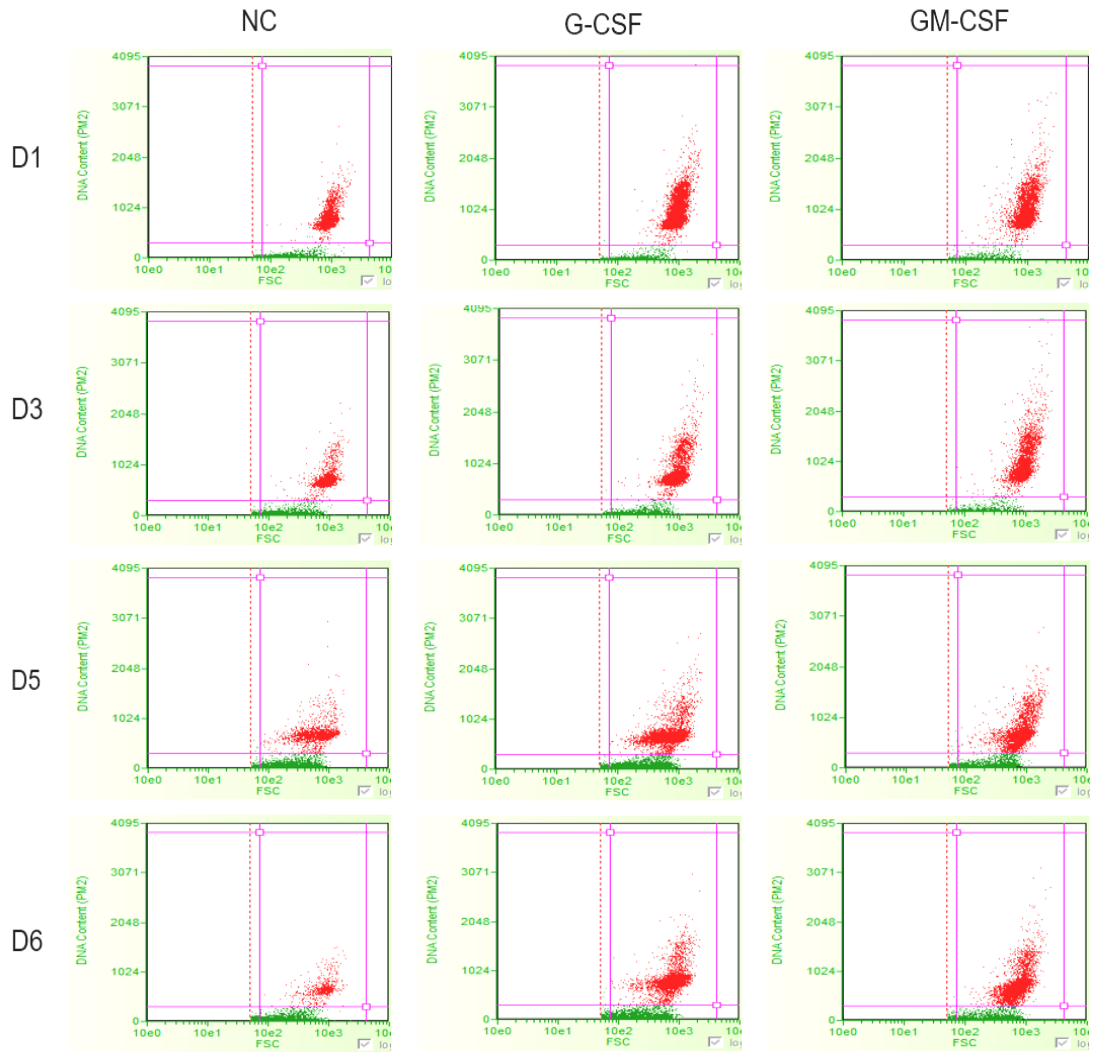


Figure 4.10 Representative flow cytometer dot plots of cell cycle parameters of dPLB-985 cells cultured with cytokine supplementation. dPLB-985 cells were incubated for 6 days in the absence (NC) and presence of cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) after media changes on days 2 and 4. Cells were stained with propidium iodide and cell cycle parameters were analysed on days 1, 3, 5 and 6 using the flow cytometer. After days 3-6 of culture there was an increase in number of cells in G0 (lower, green dots) indicating apoptotic cells. The number of which was higher after they had differentiated during culture.

4.3.4. Neutrophil cell cycle parameters

Although circulating blood neutrophils are mature, terminally-differentiated, non-dividing cells that have lost the ability to undergo cell cycle or DNA replication (Edwards, 1994), it was necessary to determine the cell cycle distribution patterns among live and apoptotic (aged) blood neutrophils, to compare with the neutrophil-like differentiated PLB-985 cells. Freshly isolated neutrophils (0 h) and those incubated with human AB serum overnight (20 h) were therefore prepared, stained with propidium iodide and cell cycle progression analysed using the flow cytometer.

As shown in Figures 4.11-12, in fresh neutrophils, most of the cells were initially accumulated in G1 phase: (70.89% ± 13.05%) with almost none in G0 (5.01% ± 2.52%). An insignificant number of cells were however, observed in S (4.50% ± 1.14%) and G2 (1.81% ± 0.42%) phases. Following 20 h incubation in culture, a significant number ($p \leq 0.05$) of cells had accumulated in G0 (25.30% ± 2.69%), indicating the development of apoptosis and death. The few cells that were observed in S and G2 phases also decreased significantly: S (2.59% ± 0.46%), G2 (1.51% ± 0.71%). These distribution patterns parallel those of differentiated PLB-985 cells at day 6 (Figure 4.11). These observations confirm the similarity between differentiated PLB-985 cells and mature blood neutrophils in regard to their DNA staining and cell cycle parameters. Representative flow cytometer traces of dot blots for DNA staining and histograms of DNA distribution profiles are shown in Figure 4.12.

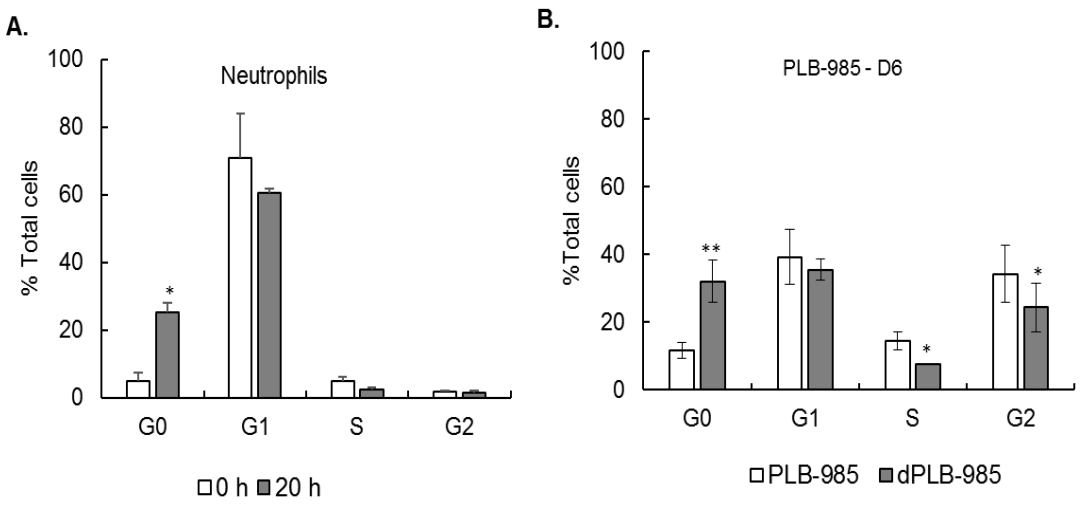


Figure 4.11 Distribution of cell cycle parameters in neutrophils and dPLB-985 cells. Freshly-isolated neutrophils (0 h) and those incubated overnight (20 h) with human AB serum (A) as well as non-differentiated (PLB-985) and differentiated (dPLB-985) cells at d 6 (B) were prepared, stained with propidium iodide and cell cycle parameters were analysed using the flow cytometer. Fresh neutrophils and PLB-985 cells were accumulated in G1, whereas aged (apoptotic) neutrophils (20 h) and dPLB-985 (day 6) both showed significant increase in G0 phase (low DNA, apoptosis) and decrease in S and G2 phases (blockage of DNA replication and inhibition of proliferation). These cell cycle parameters were calculated from the DNA distribution profiles shown in Figure 4.12. Data are expressed as percentages of total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

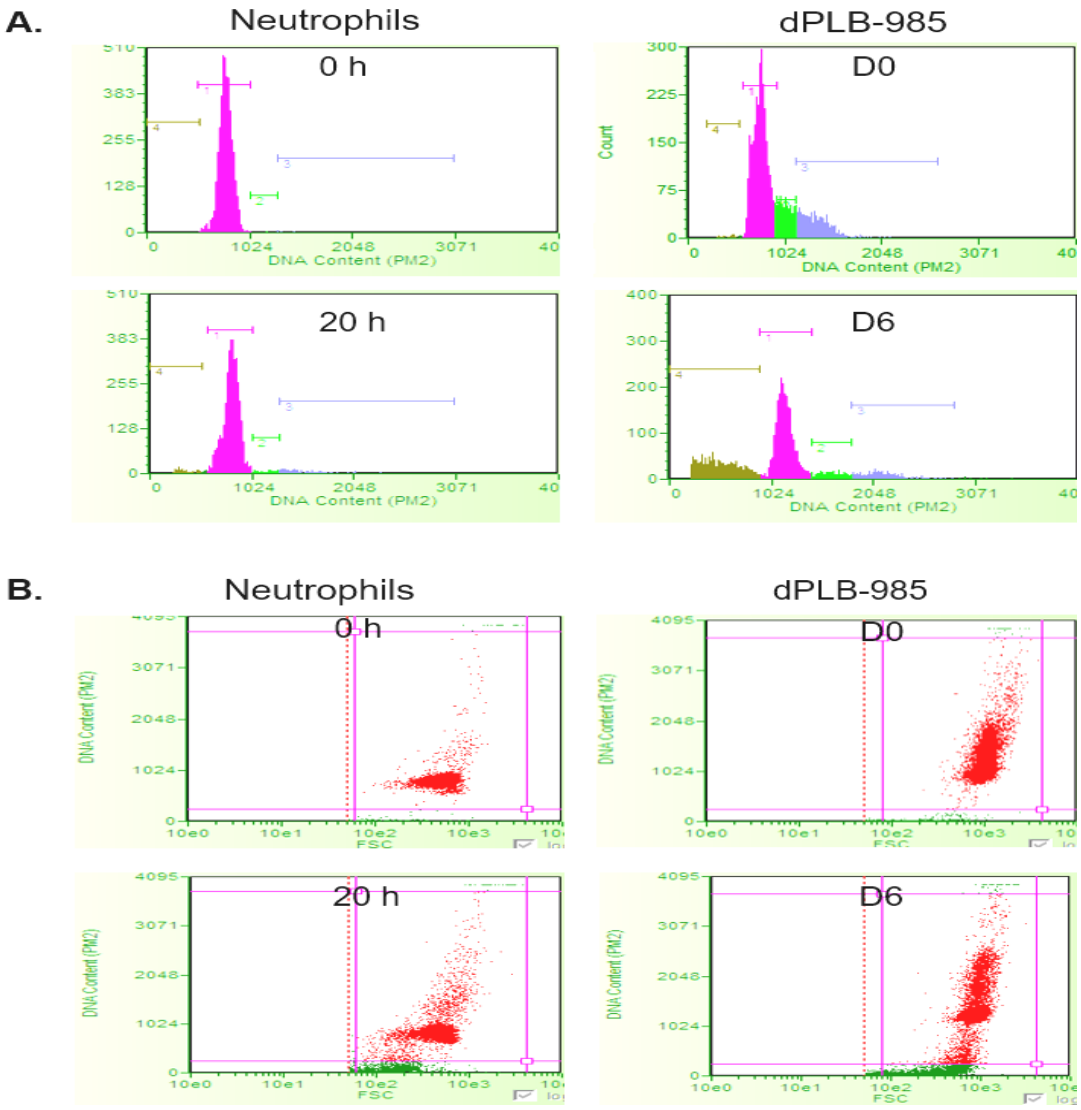


Figure 4.12 Representative flow cytometer traces of cell cycle parameters in neutrophils and dPLB-985 cells. Freshly-isolated neutrophils (0 h) and those incubated overnight (20 h) with human AB serum, as well as non-differentiated (PLB-985) and differentiated (dPLB-985) cells at day 6 were prepared, stained with propidium iodide and cell cycle parameters were analysed using the flow cytometer. (A) Flow cytometer histogram traces of DNA distribution profiles, showing accumulation of cells in G0 phases in aged neutrophils (20 h) and dPLB-985 cells. (B) Dot plots of DNA staining, showing cells with low DNA contents in G0 (lower, green dots) in aged neutrophils (20 h) and dPLB-985 cells.

4.3.5 Apoptosis of neutrophils and differentiated PLB-985 cells

Mature blood neutrophils are short-lived cells which rapidly and constitutively die by apoptosis (Edwards, 1994). To assess this, isolated blood neutrophils from healthy adult donors were incubated for 20 h with 10% human AB serum, in the absence and presence of cytokine GM-CSF (5ng/mL). Cells were then stained with Annexin V/PI and the percent apoptosis analysed using the flow cytometer.

Figure 4.12 shows the percent apoptosis in neutrophils as 73% ± 3.17% and 43% ± 2.90% in the absence and presence of GM-CSF respectively. Incubation of human neutrophils with GM-CSF significantly decreased the percent apoptosis after 20 h incubation, as detected by Annexin V staining (Figures 4.13 and 4.14). Levels of apoptosis were also confirmed by morphological examination of stained cytospin slides, prepared from fresh and aged neutrophils, viewed under a light microscope (Figure 4.15).

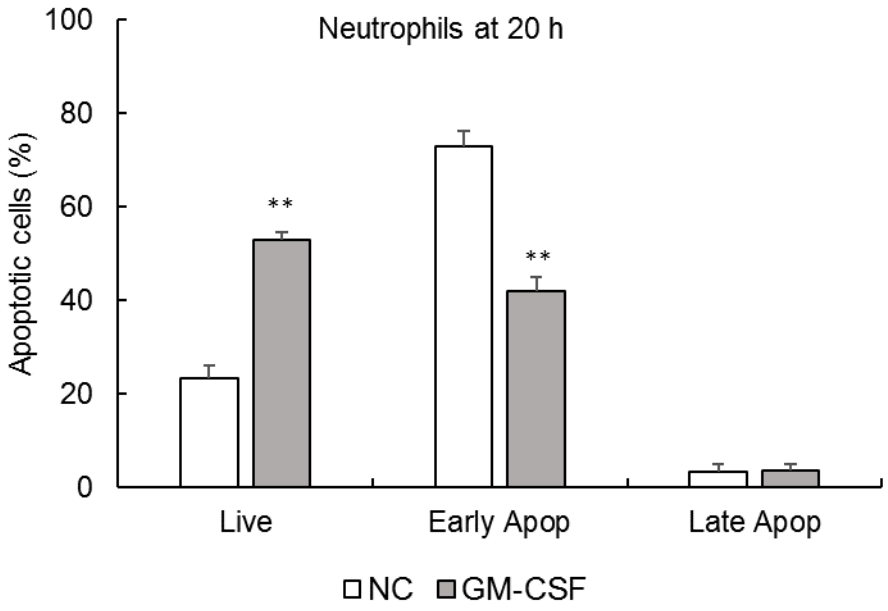


Figure 4.13 Percent neutrophil apoptosis after overnight incubation. Isolated blood neutrophils from healthy donors were incubated with 10% human AB serum for 20 h, in the absence (NC) and presence of the cytokine GM-CSF (5ng/mL). Cells were stained with Annexin V/PI, and apoptosis was analysed using the flow cytometer. Neutrophils displayed high levels of spontaneous apoptosis which was significantly decreased ($p \leq 0.01$) following incubation with GM-CSF. These apoptosis parameters were calculated from DNA staining profiles of Annexin V and PI shown in Figure 4.14. Data are expressed as percentages of total cells (± SEM, n=3), ** = $p \leq 0.01$ (paired, two-tailed student's t-test).

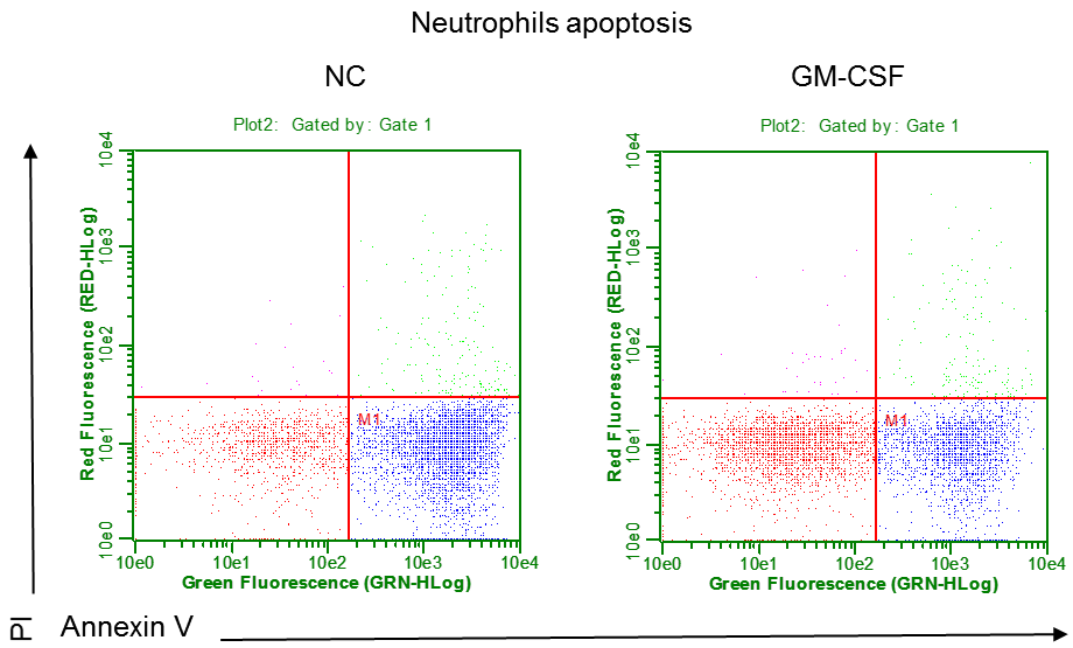


Figure 4.14 Representative flow cytometer traces of neutrophil apoptosis after overnight incubation. Isolated blood neutrophils from healthy donors were incubated with 10% human AB serum for 20 h, in the absence (NC) and presence of the cytokine GM-CSF (5ng/mL). Cells were stained with Annexin V/PI, and apoptosis was analysed using the flow cytometer. The dot plots showed distribution of neutrophils as live (lower left quadrant), early apoptotic (lower right quadrant) and late apoptotic (upper right quadrant). Cells incubated with GM-CSF showed high cell proportion in the live quadrant and low proportion of cells in the apoptosis quadrant.

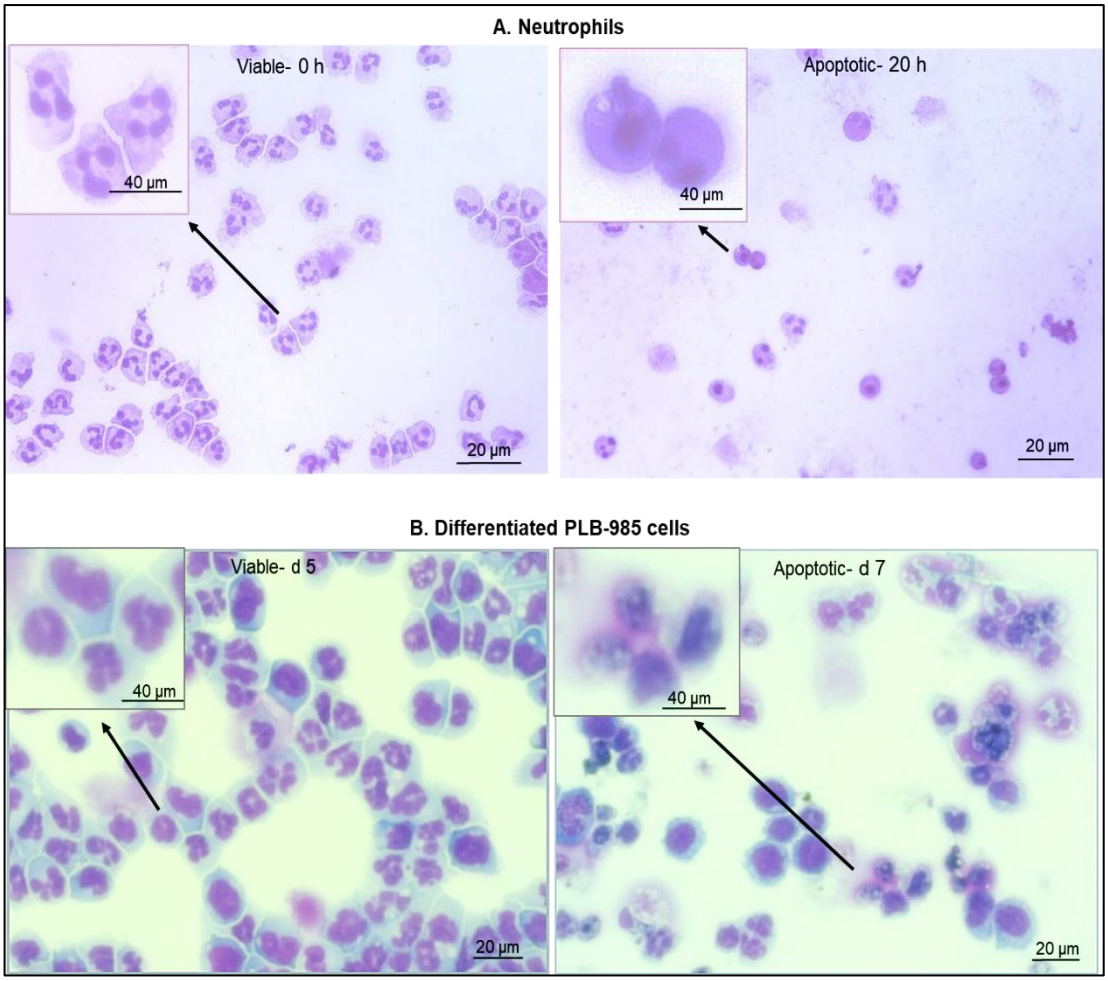


Figure 4.15 Morphology of viable and apoptotic neutrophils and dPLB-985 cells. Cytospin slides were prepared from neutrophils and differentiated PLB-985, stained with Romanowsky stain and viewed under a light microscope. (A) Freshly-isolated (0 h) and apoptotic (20 h) neutrophils. (B) Viable dPLB-985 cells (at day 6, cultured with GM-CSF as described in Figure 3.16) and apoptotic dPLB-985 cells (at day 6, cultured without cytokine). Both viable neutrophils and dPLB-985 cells exhibited indented, convoluted, and segmented nuclei, decreased nucleoli and granulated cytoplasm, whilst both apoptotic neutrophils and dPLB-985 cells appeared smaller with dense cytoplasm, condensed chromatin and had round compact nuclei (especially insets at high magnifications). Also, GM-CSF delayed apoptosis of the differentiated PLB-985 cells.

4.3.6 Effect of differentiation of PLB-985 cells on the expression of Bcl-2 family proteins

Human neutrophils do not express the anti-apoptotic proteins Bcl-2 or Bcl-X_L, but express Mcl-1, and levels of this protein correlate with apoptosis: viable cells have high Mcl-1 levels, whereas Mcl-1 levels are low in apoptotic cells (Derouet et al., 2004, Edwards et al., 2004). It was therefore important to examine the expression levels of these proteins in PLB-985 cells during differentiation into neutrophil-like cells and during apoptosis of the differentiated cells. Hence, protein extracts were prepared from PLB-985 and dPLB-985 cells cultured with media changes on days 2 and 4. Western blot analysis was performed with a range of human anti-Bcl-2 protein family antibodies, to detect the expression levels of anti-apoptotic members of Bcl-2 protein family, Mcl-1, Bcl-X_L and Bcl-2, as well as pro-apoptotic members, Bax and Bak.

4.3.6.1 Expression of anti-apoptotic Bcl-2 proteins family in PLB-985 cells

The expression of *anti-apoptotic* Bcl-2 family proteins, Mcl-1 and Bcl-X_L were measured in PLB-985 cells cultures that were incubated in the absence (PLB-985) and presence (dPLB-985) of differentiation-inducing agents. Figure 4.16 shows the relative levels of expression of Mcl-1 and Bcl-X_L in these cells with day 1 level taken as 100%. Levels of Mcl-1 in PLB-985 cells (non-differentiated) decreased after day 3 in culture but then increased up to day 6. In contrast, Mcl-1 levels increased significantly after day 1 in differentiation culture (dPLB-985) but the levels decreased markedly and significantly by day 6, when there were significant numbers of apoptotic cells in these cultures (Figure 4.16). Similar changes in levels of expression of Bcl-X_L protein were observed during culture, notably a marked decrease in the levels of this

protein as the PLB-985 cells differentiated into mature neutrophil-like cells and then underwent apoptosis by day 6.

Figure 4.17 showed similar patterns of expression levels for Bcl-2 protein in PLB-985 (non-differentiated) cells, which remained high over the culture period (D3: 52.12 ± 7.50, D5: 73.63 ± 5.92 and D6: 95.04 ± 6.10). However, changes in the level of expression of this protein were more dramatic in differentiating cells, while Bcl-2 protein was detected after day 1 in differentiation culture, it had decreased significantly from day 0 values. Also, from day 3 onwards, this protein was not detected. Thus, as PLB-985 cells differentiated into mature neutrophil-like cells, expression levels of this protein rapidly declined, which is in line with the observation that mature human neutrophils do not express Bcl-2 protein (Cross et al., 2005, Moulding et al., 1998).

4.3.6.2 Expression of pro-apoptotic Bcl-2 proteins family in PLB-985 cells

The expression levels of pro-apoptotic Bcl-2 proteins, Bax and Bak are shown in Figure 4.18. Bax and Bak proteins were expressed highly in both PLB-985 and dPLB-985 cells and their expression levels in both cultures changed very little over the 6-day period. Again, this is in line with observations from human neutrophils in which levels of these proteins are largely unaltered in either viable or apoptotic cells (Edwards et al., 2004, Moulding et al., 2001).

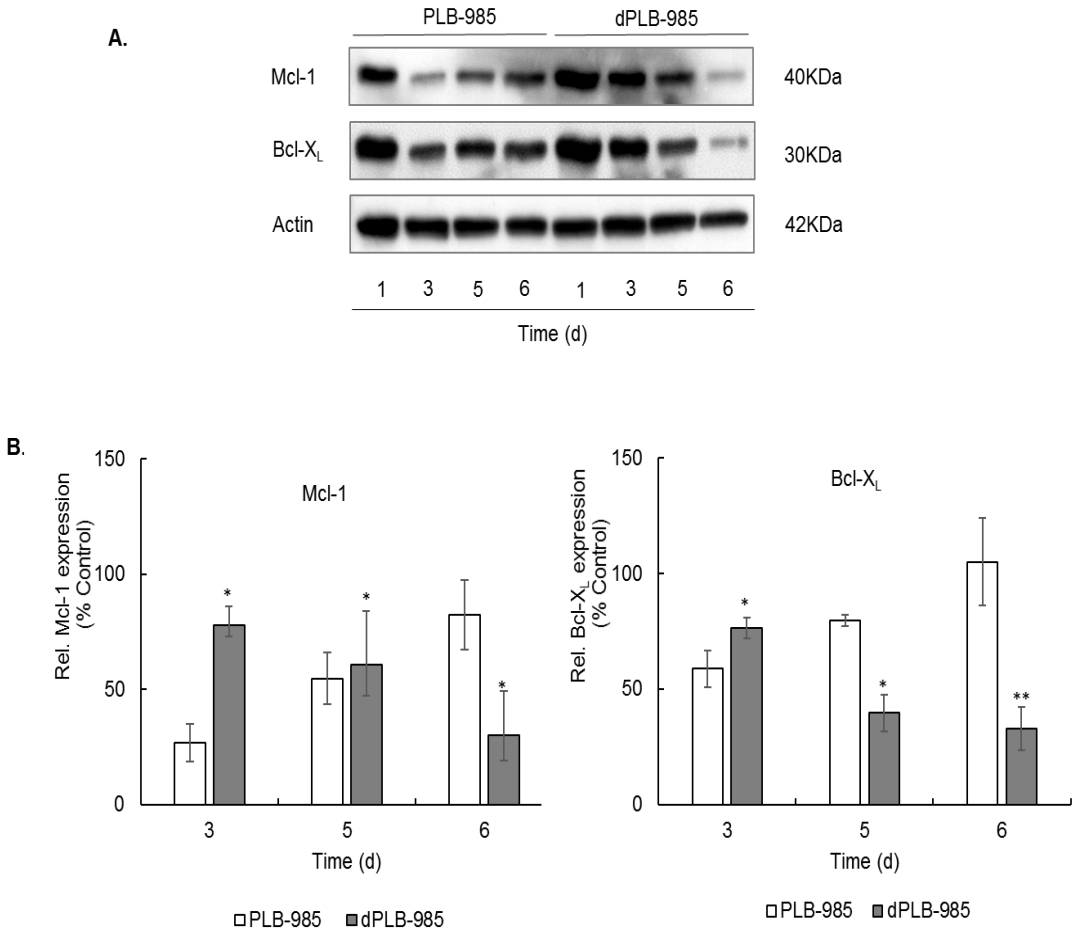


Figure 4.16 Effect of differentiation of PLB-985 cells on expression of Mcl-1 and Bcl-X_L proteins. Non-induced (PLB-985) and differentiation-induced (dPLB-985) cells were cultured for 6 d in unchanged media. Cells lysates were prepared after days 1, 3, 5, and 6 of culture and probed for Mcl-1, Bcl-X_L and Actin proteins by western blotting. (A) Western blots showing Mcl-1(40KDa), Bcl-X_L (30KDa) and Actin (42KDa) expression, representing three separate experiments. (B) Densitometric analysis of Mcl-1 and Bcl-X_L levels (normalised to the Actin signal which was the loading control). Data are expressed as percentages of day 1 values that were taken as 100% (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (two-tailed student's t-test).

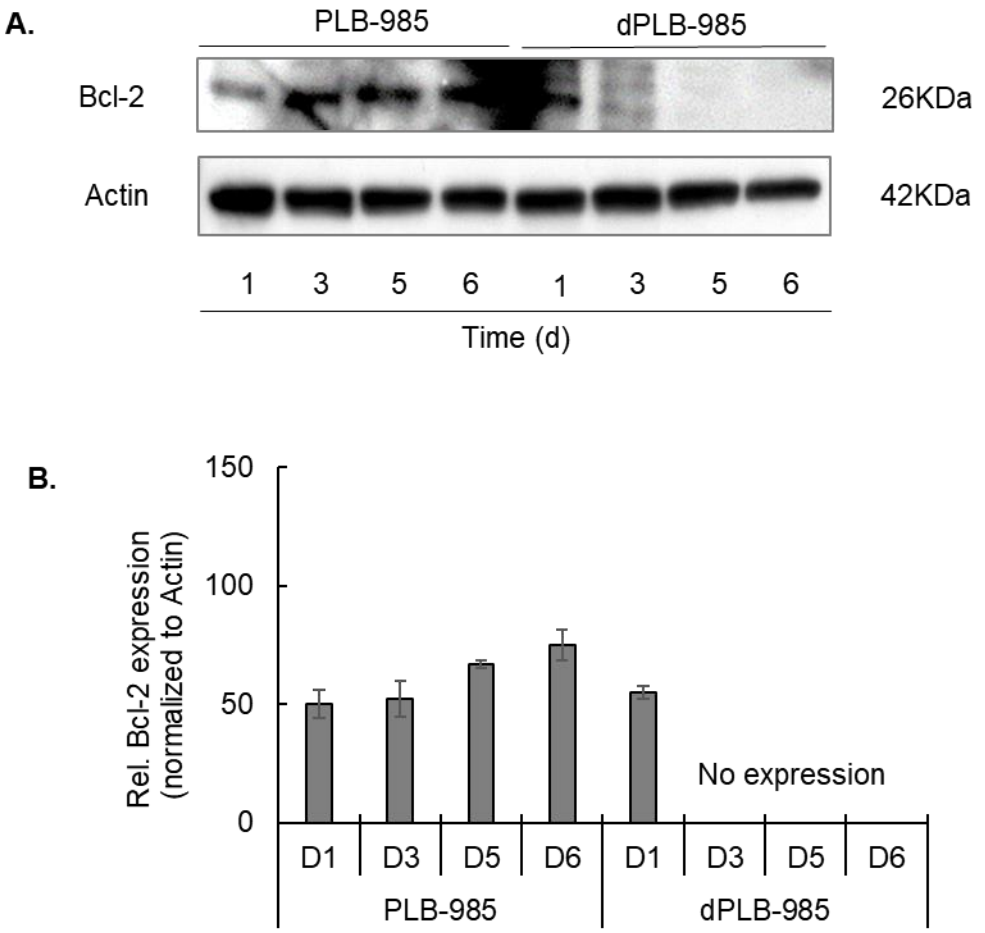


Figure 4.17 Effect of differentiation of PLB-985 cells on expression of Bcl-2 protein. Non-differentiating (PLB-985) and differentiation-induced (dPLB-985) cells were cultured for 6 days in unchanged media. Cells lysates were prepared after days 1, 3, 5, and 6 of culture and probed for Bcl-2 and Actin proteins by western blotting. (A) Western blots showing Bcl-2 (26KDa) and Actin (42KDa) expression, representing three separate experiments. (B) Densitometric analysis of Bcl-2 levels (normalised to the Actin signal which was the loading control). Bcl-2 expression was lost in dPLB-985 cells by day 3 following their differentiation into neutrophil-like cells, as blood neutrophils do not express this protein (± SEM, n=3).

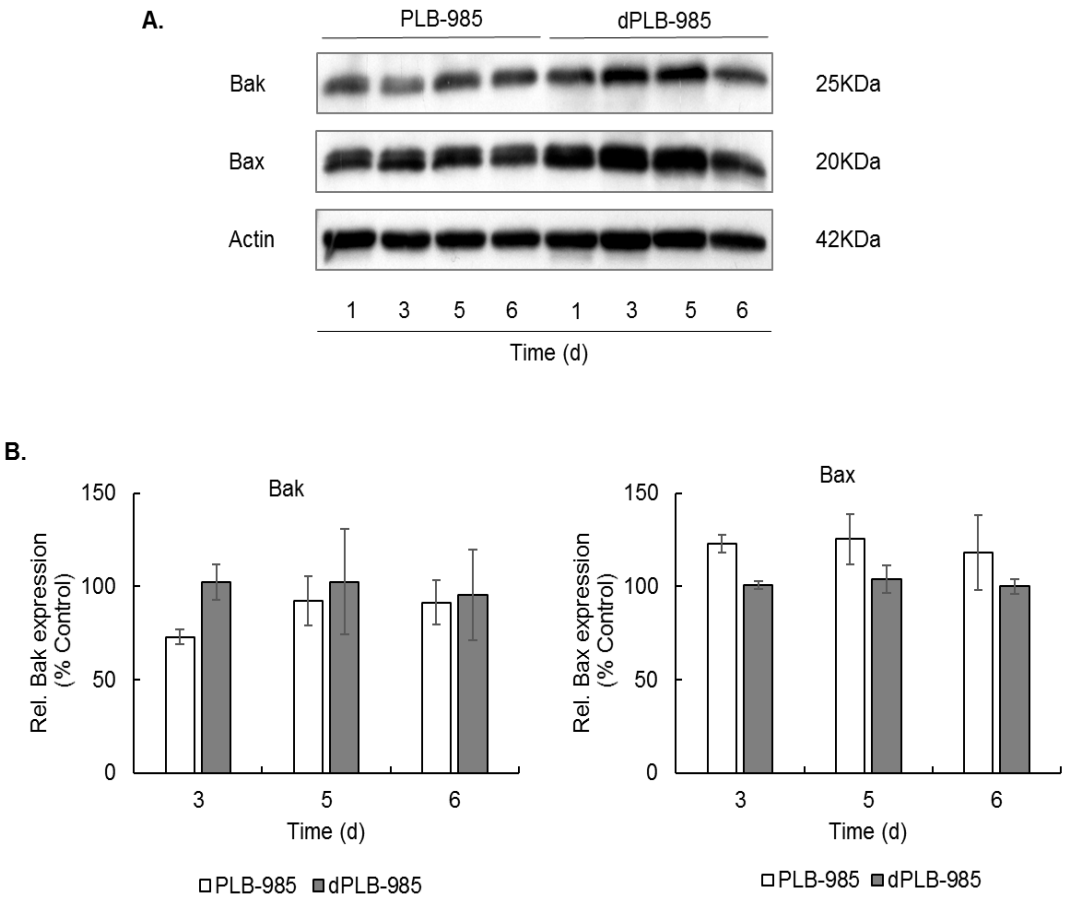


Figure 4.18 Effect of differentiation of PLB-985 cells on expression of Bak and Bax proteins. Non-induced (PLB-985) and differentiation-induced (dPLB-985) cells were cultured for 6 d in unchanged media. Cells lysates were prepared after days 1, 3, 5, and 6 of culture and probed for Bak, Bax and Actin proteins by western blotting. (A) Western blots showing Bak (25KDa), Bax (20KDa) and Actin (42KDa) expression, representing three separate experiments. (B) Densitometric analysis of Bak and Bax levels (normalised to the Actin signal which was the loading control). Data are expressed as percentages of day 1 values that were taken as 100% (± SEM, n=3).

4.3.7 Effects of media changes and GM-CSF on the expression of Bcl-2 proteins

In line with the previous finding that Mcl-1 and Bcl-X_L proteins levels correlated with differentiation and viability of the differentiated PLB-985 cells, it was then necessary to determine if supplementation of media changes with cytokine GM-CSF, which increased the differentiation levels and delayed apoptosis of the differentiated PLB-985 cells, affected expression of these proteins. GM-CSF is known to enhance the survival of mature blood neutrophils by stabilising the level of Mcl-1 protein (Edwards et al., 2004, Moulding et al., 1998). Protein lysates were prepared from PLB-985 and dPLB-985 cells cultured without (NC) and with cytokine GM-CSF (5ng/mL) added after media changes on days 2 and 4. Western blot analysis was performed to detect the expression levels of the anti-apoptotic members of Bcl-2 protein family, Mcl-1, Bcl-X_L and Bcl-2, as well as the pro-apoptotic members, Bax and Bak.

4.3.7.1 Expression of anti-apoptotic Bcl-2 proteins family

Figure 4.19 showed expression of Mcl-1 and Bcl-X_L proteins following supplementation of media changes with GM-CSF. Supplementing the media changes with GM-CSF maintained the expression of both Mcl-1 and Bcl-X_L up to day 4 (Figure 4.19), above the levels measured in the absence of GM-CSF (Figure 4.16), and levels of both these proteins were still detectable after days 5 and 6 in the differentiated PLB-985 cells, albeit at decreased levels compared to day 1. These observations are in line with measurements of cell viability and apoptosis in Chapter 3 of this thesis: there was decreased apoptosis in differentiated PLB-985 cells following media changes (Figure 3.5B) and GM-CSF supplementation (Figure 3.9). Again, Bcl-2 protein expression was not detected despite supplementation of media changes with GM-CSF addition (data not shown).

4.3.7.2 Expression of pro-apoptotic Bcl-2 proteins family

As shown in Figure 4.20, there were no significant changes in the expression levels of pro-apoptotic proteins Bax and Bak, in differentiation cultures with GM-CSF supplementation after media changes. Similarly, Bak and Bax expression levels in both cultures changed very little over 6 days period, despite supplementation of differentiation media changes with GM-CSF. This is also in line with observation that Bax expression in neutrophils remains unchanged after treatment with apoptosis delaying agents, such as GM-CSF, IL-8, LPS or butyrate (Moulding et al., 1998).

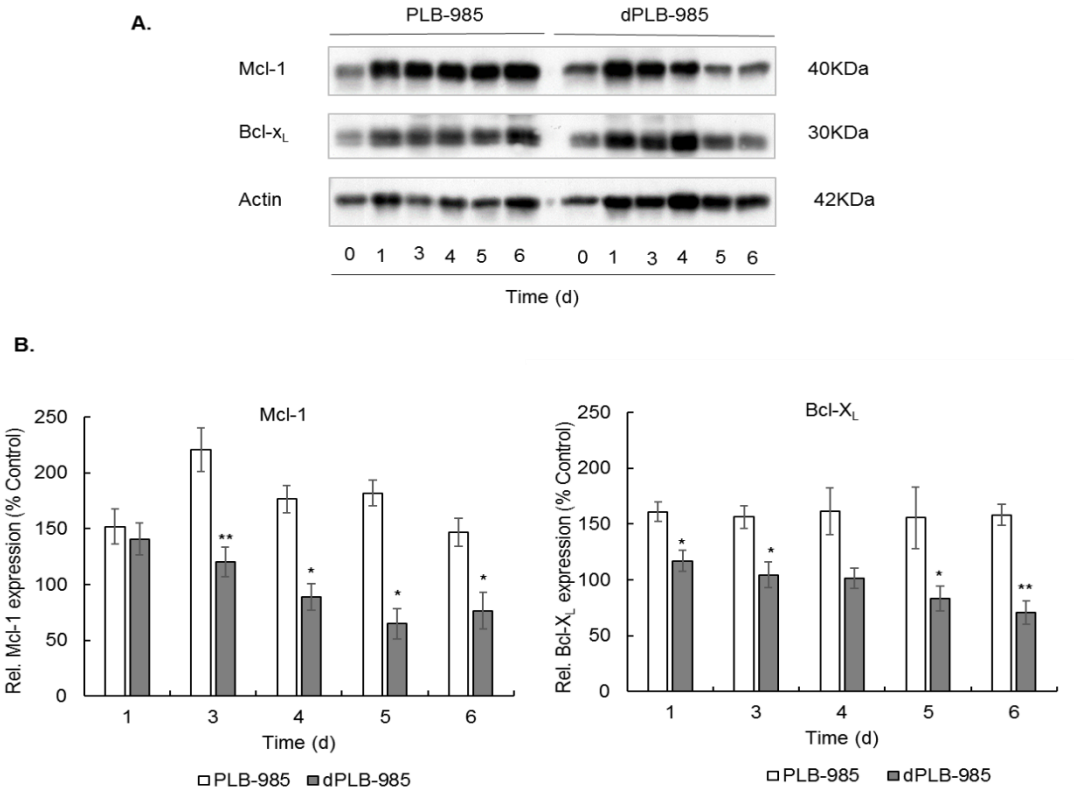


Figure 4.19 Effects of media changes and GM-CSF supplementation on expression of Mcl-1 and Bcl-X_L in dPLB-985 cells. Non-differentiated (PLB-985) and differentiation-induced (dPLB-985) cells were incubated for 6 days with GM-CSF (5ng/mL) addition after differentiation media changes on days 2 and 4. Cell lysates were prepared and probed for Mcl-1, Bcl-X_L and Actin proteins by western blotting (A) Western blots showing Mcl-1(40KDa), Bcl-X_L (30KDa) and Actin (42KDa) expression, representing three separate experiments. (B) Densitometric analysis of Mcl-1 and Bcl-X_L levels (normalised to the Actin signal which was the loading control). Data are expressed as percentages of day 1 values that were taken as 100% (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

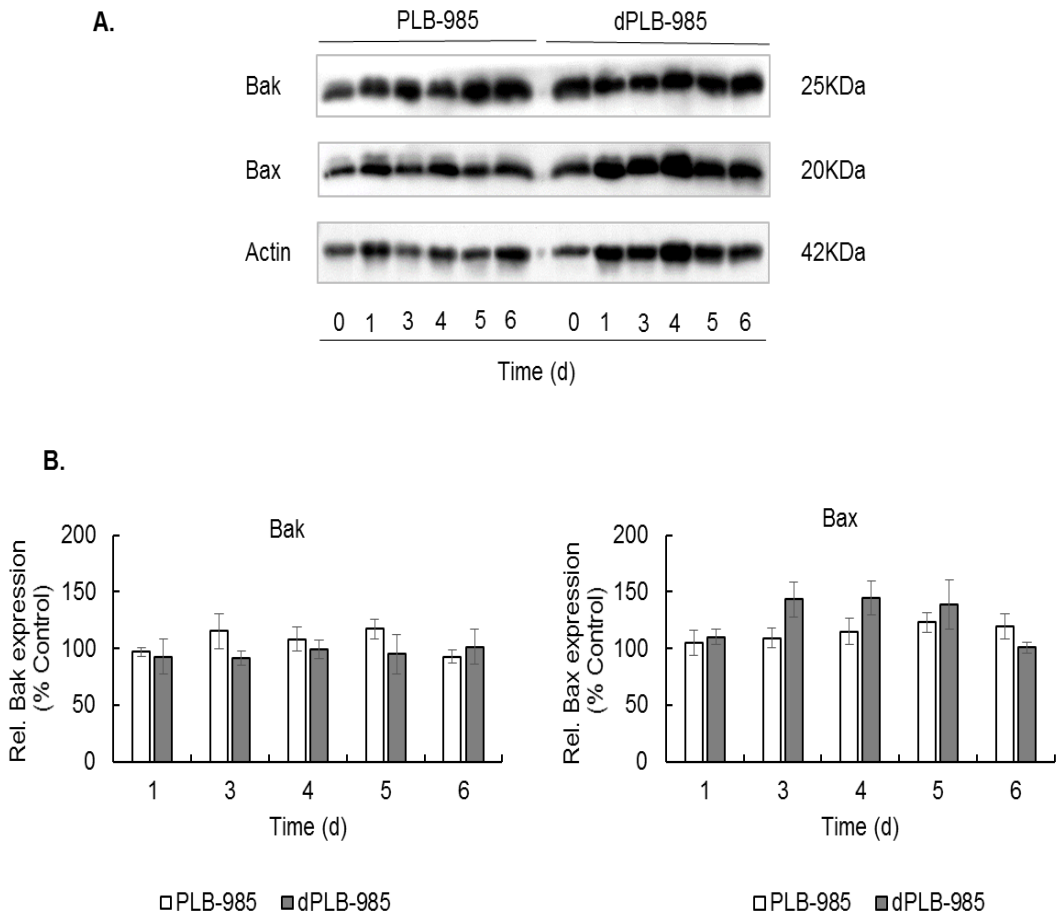


Figure 4.20 Effects of media changes and GM-CSF supplementation on expression of Bak and Bax in dPLB-985 cells. Non-differentiated (PLB-985) and differentiation-induced (dPLB-985) cells were incubated for 6 days with GM-CSF (5ng/mL) addition after differentiation media changes on days 2 and 4. Cell lysates were prepared and probed for Bak, Bax and Actin proteins by western blots. (A) Western blotting showing Bak (25KDa), Bax (20KDa) and Actin (42KDa) expression, representing three separate experiments. (B) Densitometric analysis of Bak and Bax levels (normalised to the Actin signal which was the loading control). Data are expressed as percentages of day 1 values that were taken as 100% (± SEM, n=3).

4.4 Discussion and conclusions

When extracellular signals trigger a cell to commit to undergo differentiation, the cell must recognise (via receptors) and process the signal, then activate specific transcription factors that result in changes (increases or decreases) in the expression of specific genes, which change the molecular and phenotypic properties of the cell. These changes often limit cell proliferation and the terminally-differentiated cells, in this case neutrophils, may then begin to die by apoptosis (Kim & Seoh, 2015). The fact that the same initial signal can give rise to these different cellular changes, suggests that these different events are interconnected, but how they are regulated remains to be elucidated (Yen et al., 1985).

This Chapter has measured the changes in cell cycle parameters, apoptosis and expression of key proteins that regulate apoptosis, as PLB-985 cells differentiate into neutrophil-like cells and then constitutively undergo apoptosis. It has also measured these changes when cells were incubated under conditions (described in Chapter 3), that optimised both their differentiation efficiency and survival period by delaying apoptosis. These optimised conditions involved both changing the differentiation medium and supplementation with cytokines, such as G-CSF and GM-CSF (Figure 3.16). Although data measuring the effects of G-CSF were also obtained in parallel with GM-CSF, some of the G-CSF data were not presented in this Chapter, because they were essentially identical to those obtained with GM-CSF.

The relative distribution of cells in G1, S and G2 phases of the cell cycle, as well as in G0, has been analysed after staining permeabilised, fixed cells with propidium iodide. The data presented show that induction of differentiation of PLB-985 cells initially triggers G1 arrest with little or no cells in G0, and subsequently after days 3-6 of culture, there was a significantly increased number of cells in G0 phase and fewer

cells in S phase. These observations suggest that differentiation of PLB-985 cells into mature neutrophil-like, parallels the proliferation arrest of mature neutrophils at G1. Incubation with the cytokines G-CSF and GM-CSF increased the number of differentiated cells in G0, but contrary to the cells incubated with differentiation media changes alone (without cytokine supplementation), there was a decreased number of cells in G0, following incubation with the apoptosis-delaying cytokines, G-CSF and GM-CSF. Therefore, these observations confirm that these cytokines prolong the survival of the differentiated cells. In addition, there was a decrease in the number of cells in S and G2 phases, indicating that these cytokines have not reversed the effect of differentiation in inhibiting DNA synthesis and cell proliferation of differentiated PLB-985 cells.

These results are consistent with the findings of Fibach, *et al*., who reported loss of proliferation capacity in HL-60 after 2-3 divisions in response to other differentiation inducers (Fibach et al., 1982). The accumulation of differentiated PLB-985 cells in G1 means that these cells were arrested at G1/S and do not progress to the G1-to-S transition (Yen et al., 1985). Moreover, the G2/M arrest was observed which blocked their entry into mitosis, since this checkpoint is activated upon blockage of DNA synthesis and/or prevention of segregation of damaged or incompletely synthesized DNA (Pucci et al., 2000). These findings suggest that differentiation of PLB-985 into neutrophil-like cells triggers their growth arrest and progression into apoptosis.

DNA distribution profiles of freshly-isolated blood neutrophils shown in Figures 4.11-12, reveal that most of the cells were arrested in G1 phase, with little or none in G0 phase. An insignificant number of cells were however, observed in the S and G2 phases, which could have arisen from contaminating monocytes or lymphocytes in the neutrophil preparations, as it is a known fact that mature blood neutrophils do not undergo DNA replication or proliferation. Following a 20 h incubation however, a

significant number of cells accumulated in the G0 phase, indicative of apoptosis and/or cell death. The few cells that were observed in S and G2 phases in freshly-isolated cells also decreased after 20 h culture. These distribution patterns are similar to that of differentiated PLB-985 cells at day 6: decrease in S phase and increase in G0 phase. Thus, these experiments confirm that terminally-differentiated PLB-985 cells acquired some of the properties of blood neutrophils in respect to their cell cycle DNA distribution patterns.

Aged neutrophils (20 h) and differentiated PLB-985 cells (day 6 of incubation without media changes and/or cytokines supplementation) exhibited morphological characteristics of apoptotic cells (Elmore, 2007; Vousden, 2001). Both types of cells acquired a blebbed nuclear membrane structure, became rounded, had shrunk due to cytoplasmic condensation, and had a condensed chromatin. These findings further suggest that they both underwent spontaneous apoptosis.

The effect of differentiation of PLB-985 cells on the expression of anti- and pro-apoptotic proteins of the Bcl-2 family were investigated to determine whether changes in their expression levels may parallel and correlate with differentiation and apoptosis. Differentiation of PLB-985 cells was accompanied by a progressive decrease in expression of the anti-apoptotic proteins, Mcl-1 and Bcl-X_L levels. These findings indicate that Mcl-1 and Bcl-X_L levels correlated with the survival of differentiated PLB-985 cells as well as support the reports that Mcl-1 plays crucial role in the apoptosis of human blood neutrophils (Edwards et al., 2004, Moulding et al., 1998). Transfection studies have confirmed the role of Mcl-1 as an anti-apoptotic protein whose overexpression results in prolonged survival of cells (Reynolds et al., 1994). Emerging evidence also shows that enhanced overexpression of Mcl-1 confers a malignant cell phenotype. For example, Mcl-1 overexpression has been implicated in the resistance

to chemotherapy and impaired apoptosis of malignant myeloma cells (Zhang, et al., 2002, Okaro, et al., 2001).

Interestingly, and contrary to human blood neutrophils, which do not express detectable levels of Bcl-X_L protein by immunoblots (Edwards et al., 2004, Moulding et al., 1998), differentiated PLB-985 cells expressed detectable levels of this protein, but like Mcl-1, levels decreased as the PLB-985 differentiated into neutrophil-like cells. Although, mRNA of Bcl-X_L has been detected in human neutrophils, they do not express Bcl-X_L protein (Moulding et al., 2001). Incubation of differentiating PLB-985 cells in the absence of the pro-inflammatory agent GM-CSF, led to decreased expression levels of Mcl-1 and Bcl-X_L by day 5 and 6 in culture in line with the differentiation, followed by apoptosis. However, in the presence of GM-CSF, and in line with the ability of this cytokine to delay apoptosis, levels of these two proteins, although decreased compared to levels at earlier time points, were increased at this time, above levels observed in the absence of the cytokine. The increased levels of these two proteins therefore, parallels the delayed apoptosis in the differentiated PLB-985 cells. This result agrees with observations that GM-CSF considerably delays apoptosis of neutrophils and maintains Mcl-1 levels (Derouet et al., 2004, Moulding et al., 1998).

Non-differentiated PLB-985 cells expressed anti-apoptotic protein Bcl-2, and another striking finding was that, like mature blood neutrophils (Edwards et al., 2004), expression of Bcl-2 was rapidly lost following initiation of differentiation of PLB-985 into mature neutrophil-like cells. Many studies also reported the absence of Bcl-2 in mature blood neutrophils (Hsieh et al., 1997, Liles & Klebanoff, 1995, Lagasse & Weissman, 1994). Furthermore, these results are consistent with the findings by Okaro, *et al*. that inhibition of apoptosis by Mcl-1 and Bcl-X_L, but not Bcl-2 was

responsible for the prolonged survival of both normal and tumorigenic cells in the biliary tree (Okaro et al., 2001).

The pro-apoptotic proteins Bax and Bak, which are constitutively expressed by human neutrophils (Edwards et al., 2004) were also highly expressed by differentiated PLB-985 cells, but their levels did not significantly change during proliferation, differentiation or apoptosis of these cells. Levels of these proteins were unchanged also, after incubation with the apoptosis-delaying agent, GM-CSF. The Bcl-2 associated X protein (Bax) has been described to promote apoptosis through heterodimerizing with Bcl-2 protein, thereby counteracting the death repression activity of the latter (Oltvai, et al., 1993). Bax is cleaved by Calpain-1 into an 18kDa fragment incapable of interacting with Bcl-2 family members following its translocation to the mitochondria, thereby facilitating apoptosis (Altznauer et al., 2004, Maianski et al., 2002). Rossi *et al*. reported that *in vivo* inhibition of Bax cleavage can result in enhanced inflammation, suggesting the role of this protein in the resolution of acute inflammation (Rossi et al., 2006). Moreover, studies by Ohla *et al*. demonstrated an enhanced expression of Bcl-2 and Bax proteins in T-cells cultured with IL-2 *in vitro*. However, elevated levels of Bax, but not Bcl-2 were maintained following removal of IL-2, indicating that Bax expression is constitutive and independent of cell survival status (Ohta et al., 1995).

The Bcl-2 homologous antagonist/killer protein (Bak) has also been reported to show strong immunoreactivity in terminally-differentiated granulocytes and that through its tissue-specific expression, it regulates apoptosis at specific stages of cell differentiation (Krajewski, et al., 1996). Bazzoni *et al*., reported constitutive expression of Bak in human neutrophils and levels were not modulated irrespective of the cell's survival in culture (Bazzoni et al., 1999). Therefore, Bak expression like that of Bax, is constitutive and independent of cell's survival status. Taken together,

these reports support my findings that differentiation of PLB-985 cells did not result in any significant change in the expression levels of the pro-apoptotic proteins Bax and Bak.

In summary, the data presented in this Chapter have revealed some important findings. First, neutrophil-like differentiated PLB-985 cells expressed Bcl-X_L protein, which is not detected in neutrophils by immunoblots and which, like Mcl-1, correlates with the cell's survival. Second, non-differentiated PLB-985 cells expressed Bcl-2 protein which is absent in human mature blood neutrophils, but this expression of Bcl-2 was lost following differentiation of PLB-985 into mature neutrophil-like cells. This lends credence to the use of differentiated PLB-985 cells as a neutrophil model to understand more insights into neutrophil functional properties, such as regulation of apoptosis and cell cycle progression.

In conclusion, the experiments described in this Chapter indicate that differentiation of PLB-985 cells triggers cell cycle arrest and progression into apoptosis, and suggests possible interconnection between the two processes. Differentiated PLB-985 cells resembled isolated mature blood neutrophils with regards to their cell cycle parameters and apoptosis. Apoptotic neutrophils and differentiated PLB-985 cells exhibited similar morphological characteristics. Furthermore, apoptosis and survival of differentiated PLB-985 cells might be controlled by the differentiation-induced expression of anti-apoptotic proteins Mcl-1 and Bcl-X_L and possibly, Bcl-2 depletion. However, similar to blood neutrophils the expression levels of pro-apoptotic proteins Bax and Bak, remain largely unchanged during proliferation, differentiation and apoptosis.

Chapter 5: Changes in phagocytosis and oxidative burst activity during differentiation of PLB-985 cells

5.1 Introduction

Phagocytosis is the process of uptake of relatively large particles (≥0.5μm) by phagocytic cells. It is an essential component of the innate immune response by which cells recognise, ingest and destroy invading microorganisms, such as bacteria or fungi. Phagocytosis is also a fundamental element of tissue homeostasis and remodelling, where it is involved in the clearance of apoptotic bodies formed from the daily turnover of billions of cells (Flannagan et al., 2012). Myeloid cells, such as neutrophils, macrophages, monocytes and dendritic cells function in innate immune responses and perform professional phagocytosis to clear pathogens from the site of infection (Wu et al., 2009, Thedrez et al., 2007).

Phagocytosis is usually a receptor-mediated process whereby receptors on the cell surface of the phagocytes recognise and bind the foreign bodies either directly via pattern-recognition receptors (PAMPs) or indirectly via opsonic receptors before internalisation. It culminates with the formation of membrane-bound vacuole called the phagosome (Flannagan et al., 2012). The nascent phagosome itself is insufficient to mediate pathogen killing until a maturation process occurs, which transforms the phagosome into a potent cytotoxic phagolysosome that is acidic, oxidising and rich in hydrolase and protease enzymes. This transition involves influx and efflux of materials through the fusion of phagosomes to endosomes initially, and subsequently to lysosomes, thereby acquiring functions of both the endosome (protein recycling) and the lysosome (degradative functions) (Pitt et al., 1992). In neutrophils, the azurophilic and specific granules fuse with the newly-formed phagosome and via this

process, the ingested particle is exposed to a host of cytotoxic and degradative enzymes, such as myeloperoxidase, proteases and hydrolases (Edwards, 1994).

The oxidative burst is a metabolic process that occurs during phagocytosis through which phagocytes produce reactive oxygen species (superoxide and hydrogen peroxide) that are either directly or indirectly toxic to the pathogens (Kim & Seoh, 2015). Pathogen uptake triggers ROS generation following fusion of cytoplasmic granules (degranulation) leading to phagosome formation. Within the phagosome, ROS are generated by an activated membrane bound NADPH oxidase and cytotoxic proteins are released from the granules into the phagocytic vesicle, that kill and degrade the engulfed pathogen (Kennedy & Deleo, 2009). The multi-component enzyme complex, NADPH oxidase becomes assembled and activated and causes the formation of ROS from oxygen inside the phagosome (see Figure 1.4) (Edwards, 1996).

The NADPH oxidase complex is composed of at least 7 proteins that reside on the plasma membrane and membrane of specific granules and in the cytosol of resting neutrophils. Upon activation, e.g. via phagocytosis, the cytosolic proteins translocate to the phagosome membrane where they associate with the membrane proteins to assemble the NADPH oxidase. The assembled NADPH oxidase catalyses the formation of superoxide (O_2^-) by electron transfer from cytosolic NADPH to O_2, which then rapidly dismutates into H_2O_2. A granule protein, myeloperoxidase (MPO) catalyses the reaction of hydrogen peroxide (H_2O_2) with chloride ions to form hypochlorous acid (HOCl) and other secondary derived ROS, such as the hydroxyl radical ($OH^{\cdot}$) and singlet oxygen (O^-) (Kennedy & Deleo, 2009, Quinn et al., 2006).

The capacity of differentiated PLB-985 cells to undergo phagocytosis and ROS generation would therefore be a good indication of their ability to have acquired the

properties of mature neutrophils. Martinez, *et al*., demonstrated that *in vitro* differentiated HL-60 cells undertook phagocytosis of labelled pneumococci in a similar manner to that of isolated blood phagocytes (Martinez et al., 1999). Several other studies involving differentiated leukaemia cell lines into granulocytes, under a variety of differentiation conditions reported that the differentiated cells displayed a range of phagocytic capacities (Pivot-Pajot et al., 2010, Kim & Seoh, 2015, Bissonnette et al., 2008, Pessach & Levy, 2002). The studies described in this Chapter were therefore performed to investigate phagocytosis and oxidative burst activity of blood neutrophils and neutrophil-like dPLB-985 cells, differentiated under the optimised culture conditions described in Chapters 3 and 4.

The aims of the work in this Chapter, therefore were to:

1. Determine the phagocytosis capacity of neutrophils and differentiated PLB-985 cells by flow cytometry.
2. Analyse and compare the generation of oxidative burst activity by neutrophils and differentiated PLB-985 cells.
3. Investigate the time course of differentiation of PLB-985 cells on phagocytosis and ROS production capacity, under conditions in which apoptosis of neutrophil-like cells was delayed.

5.2 Methods

To assay phagocytosis of blood neutrophils and differentiated PLB-985 cells, live *S. aureus* were heat-inactivated, PI-labelled and opsonised with human AB serum as described in detail in Chapter 2. Fluorescent red and non-fluorescent polystyrene latex beads (1.0µm) were also opsonised with human AB serum. Both SAPI and latex

beads were adjusted to the working concentrations to give cell: particle ratios of 1:10. Cells were incubated without and with priming agents, G-CSF (10ng/mL) or GM-CSF (5 ng/mL) for 30 min, followed by addition of SAPI or latex beads at 1:10 (cell to particle ratio), for further 30 min. DHR 123 (5µM) was added to the non-fluorescent latex beads for 15 min, to measure the intra-phagosomal ROS production following their internalisation, and unstained or unstimulated cells were included for negative fluorescence/control. Samples were prepared as described in Chapter 2 and phagocytosis of bacteria or latex beads by neutrophils and by differentiated PLB-985 cells was measured using the flow cytometer.

Flow cytometric analysis and luminol-enhanced chemiluminescence assays were used to measure the oxidative burst activity. For flow cytometry, neutrophils or differentiated PLB-985 cells were incubated with and without priming agents; G-CSF (10ng/mL) and GM-CSF (5 ng/mL) for 30 min, followed by addition of DHR 123 (5µM) for 15 min, as the ROS detection probe. Oxidative burst activity was then stimulated by a receptor-dependent agonist, fMLP (1µM), and a receptor-independent agonist, PMA (0.1µg/mL), or inert, non-fluorescent latex beads at 1:10 (cell to particle ratios). Dihydrorhodamine 123 (DHR 123) is a non-fluorescent dye which is permeable to cells, and is oxidised to fluorescent rhodamine 123 by the generated ROS that crosses the plasma membrane or those that are generated within the phagolysosomes (Henderson & Chappell, 1993). ROS generation by neutrophils and differentiated PLB-985 cells was measured using the flow cytometer.

For the luminol-enhanced chemiluminescence assay, neutrophils or differentiated PLB-985 cells were incubated with and without priming agents; G-CSF (10ng/mL) or GM-CSF (5ng/mL) and the oxidative burst was stimulated with fMLP (1µM), PMA (0.1µg/mL) or unstimulated (control). Samples contained luminol (10µM) and photon emission was measured in a white, low adhesion 96-well plates using a luminometer.

Luminol passes through the cell membrane and becomes oxidised by the generated ROS, releasing energy in the form of light. Luminol enhanced luminescence therefore, measured both intra and extra-cellular oxidants. The total ROS generation was measured using FLUOstar Omega plate reader from BMG Labtech.

5.3 Results

5.3.1 Neutrophils phagocytosis of SAPI and latex beads

Before the properties of differentiated PLB-985 cells could be characterised, it was first necessary to determine the phagocytic and oxidative burst properties of mature blood neutrophils. Isolated neutrophils from healthy donors, incubated without and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL), were therefore incubated with *S. aureus*-PI (SAPI), fluorescent-red beads (F-Beads) and non-fluorescent beads, containing DHR123 (NF-B + DHR) and phagocytosis was measured using the flow cytometer.

As shown on Figure 5.1A neutrophils displayed high fluorescence after phagocytosis of SAPI (MFI 236.24 ± 4.43) which was significantly increased ($p \leq 0.05$) by incubation with both G-CSF (MFI 283.55 ± 1.69) and GM-CSF (MFI 322.00 ± 13.64). Fluorescent latex beads showed moderate phagocytosis (MFI 242.34 ± 30.49) which also significantly increased ($p \leq 0.05$) after incubation with G-CSF (MFI 286.32 ± 10.59) and GM-CSF (MFI 317.58 ± 4.61). Non-fluorescent beads showed lowest response (MFI 29.72 ± 3.12) as measured by oxidation of DHR 123 by generated ROS. A slight and an insignificant increase was observed following incubation with G-CSF (MFI 30.94 ± 1.40) and GM-CSF (MFI 39.52 ± 1.44).

Figure 5.1B shows the phagocytic ability of neutrophils (% positive cells) to SAPI (93.85% ± 1.70%), which increased when incubated with G-CSF (96.14% ± 0.82%) and GM-CSF (94.79% ± 2.62%). Fluorescence after uptake of fluorescent beads (23.63% ± 0.88%) increased significantly ($p \leq 0.05$) following G-CSF (24.71% ± 0.64%) and GM-CSF (37.37% ± 2.43%) treatments, but not all cells showed a positive response. Conversely, there were more cells that stained positive after uptake of non-fluorescent beads (42.67% ± 0.73%) and this increased following G-CSF (54.26% ± 0.63%) and GM-CSF (91.94% ± 0.66%) treatments. Representative traces of neutrophil phagocytosis under these conditions are shown in Figure 5.2. The difference in fluorescence intensity is due the fact that while most neutrophils phagocytosed the non-fluorescent beads, the level of fluorescence obtained was dependent on the level of oxidation of the ROS probe, DHR 123.

Closer analysis of the positive fluorescence traces obtained after uptake of fluorescent beads indicated complexes patterns that could be explained by uptake of different numbers of particles per cell (Figure 5.2, right hand traces). Comparison of the fluorescence of latex particles (in the absence of cytokines) indicate that the individual “peaks” in fluorescence were due to uptake of different numbers of particles per cell. This analysis is shown in Figure 5.3 and shows that most neutrophils took up 2 particles per cell. Neutrophils incubated with the GM-CSF took up more particles per cell, but this did not reach statistical significance.

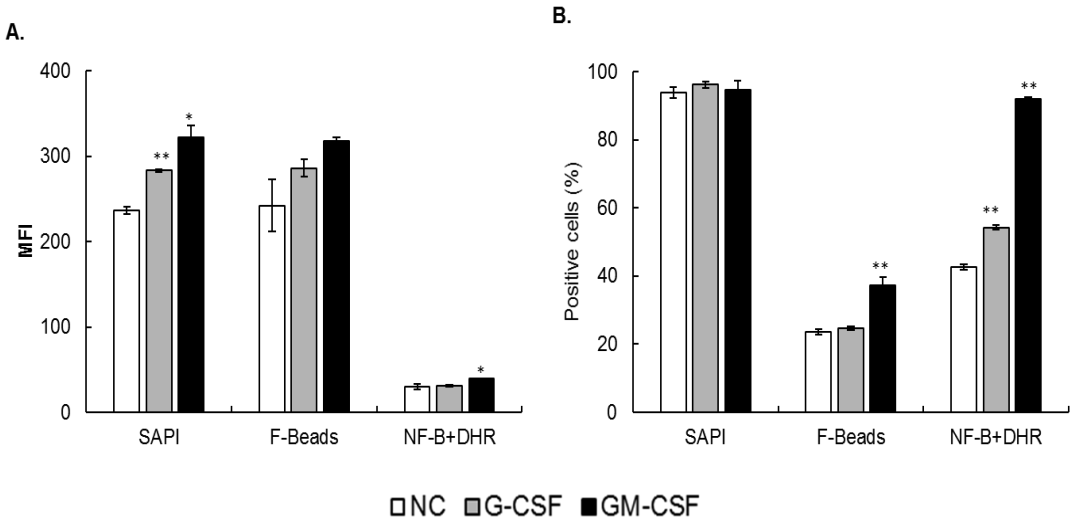


Figure 5.1 Neutrophil phagocytosis of SAPI and latex beads. Freshly-isolated blood neutrophils were incubated without (NC) and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of SAPI or latex beads at 1:10 (cell to particles ratio) for a further 30 min. DHR 123 (5µM) was added to the non-fluorescent beads (NF-B+DHR) for 15 min, and unstimulated cells (no cytokine, no particles) were included to gate for negative fluorescence. Phagocytosis was then measured using the flow cytometer. (A) Mean fluorescence intensity (MFI) of neutrophil phagocytosis (B) Percent positive cells for phagocytosis. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

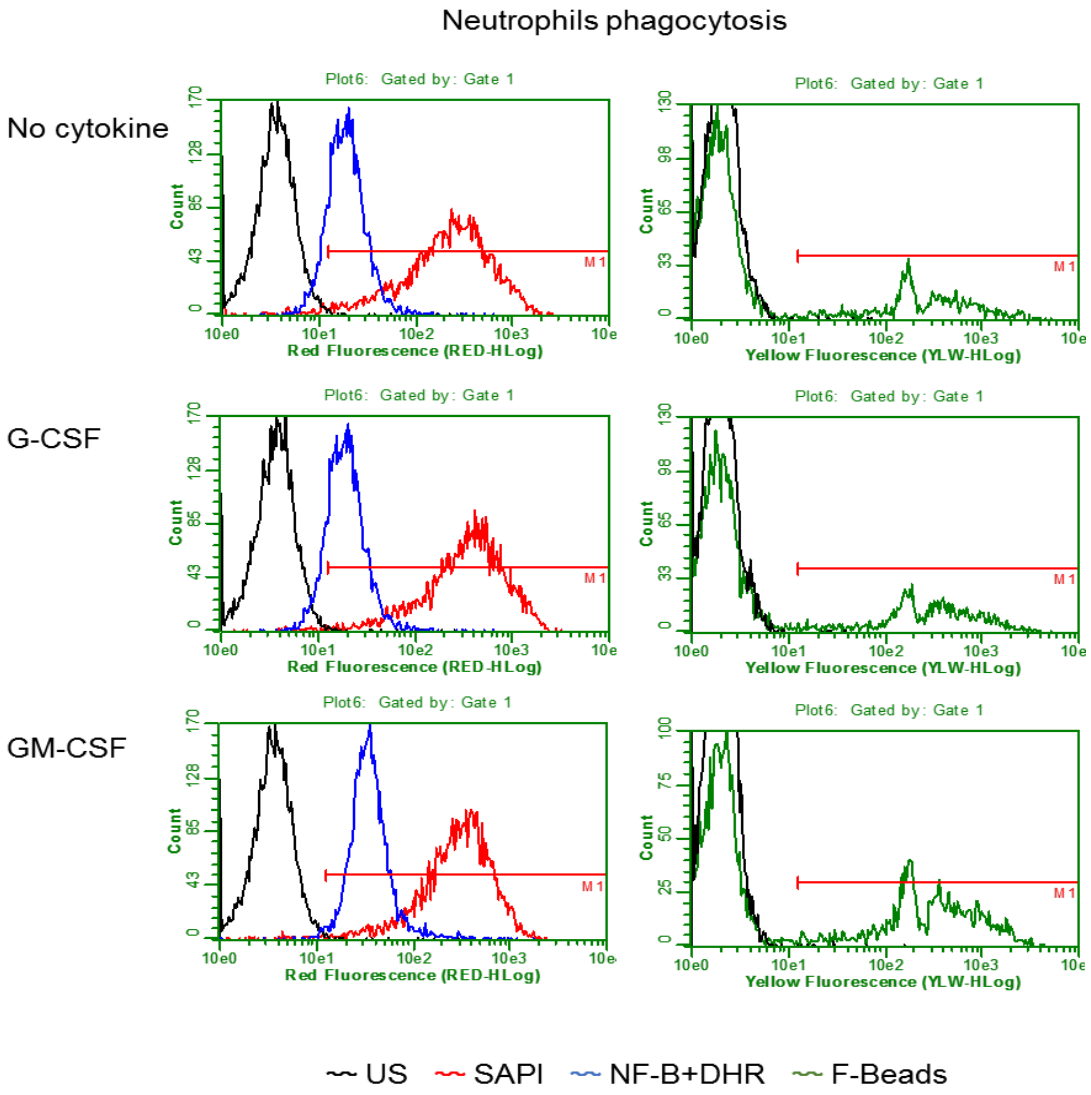


Figure 5.2 Representative traces of flow cytometer histogram for neutrophils phagocytosis of SAPI and latex beads. Freshly-isolated blood neutrophils were incubated without (NC) and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of SAPI or latex beads at 1:10 (cell to particles ratio) for further 30 min. DHR 123 (5µM) was added to the non-fluorescent beads (NF-B+DHR) for 15 min. Phagocytosis was then measured using the flow cytometer. Black traces (US) indicate unstimulated cells (no particles) used to gate for negative fluorescence. Red traces (SAPI) and blue traces (NF-Beads + DHR) were measured in the red fluorescence channel while green traces (F-Beads) were measured in the yellow fluorescence channel.

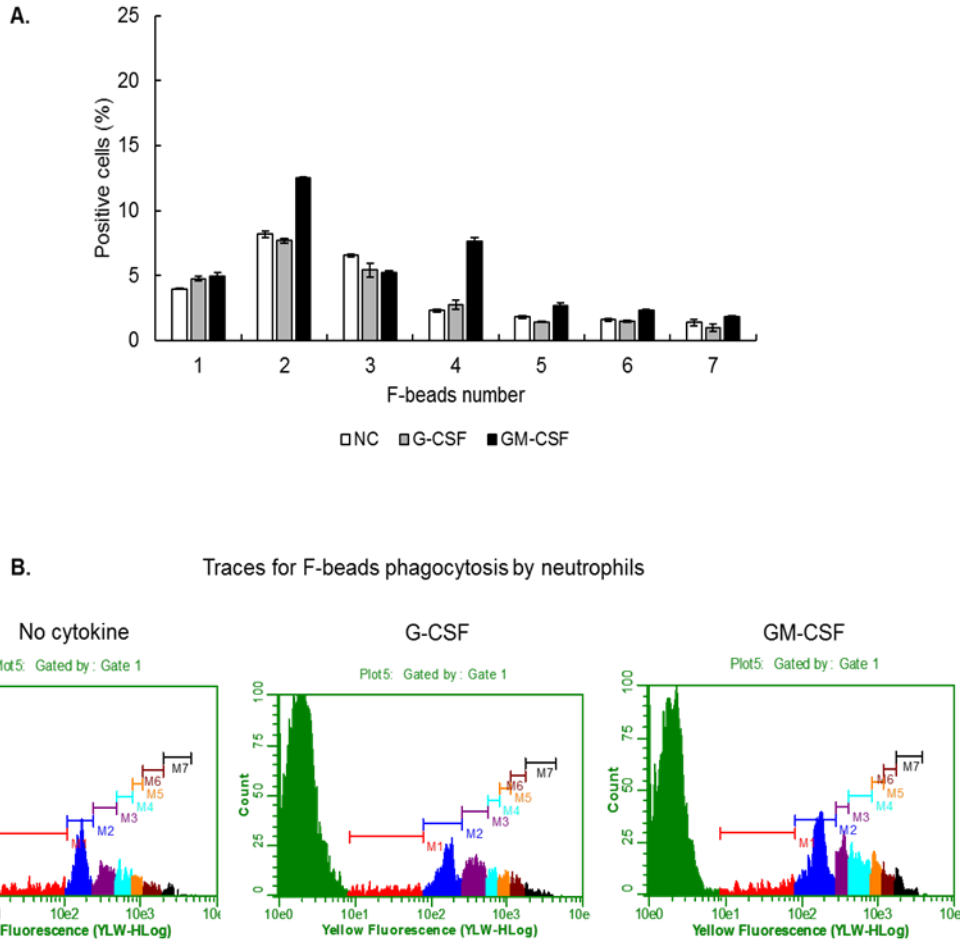


Figure 5.3 Proportions of neutrophils that phagocytosed different numbers of fluorescent latex beads. Freshly-isolated blood neutrophils were incubated without and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of the fluorescent beads at 1:10 (cell to particles ratio) or unstimulated (no particles) to gate for negative fluorescence, for a further 30 min. Phagocytosis was then measured using the flow cytometer. (A) Percentages of cells that engulfed I, 2, 3, 4, 5, 6 and 7 beads, under the different treatments, calculated from Figure 5.3B. (B) Representative flow cytometer traces for proportion of positive cells that engulfed 1-7 numbers of beads (M1-M7). Green traces indicate cells that did not take up any particle. Data are expressed as means of percent positive cells (± SEM, n=3).

5.3.2 Differentiated PLB-985 cells phagocytosis of SAPI and latex beads

PLB-985 cells were differentiated under the optimised conditions, involving media changes and cytokine addition as described in Figure 3.16. Samples of dPLB-985 cells, incubated with and without cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) were incubated with *S. aureus*-PI (SAPI), fluorescent-red beads (F-beads) and non-fluorescent beads with DHR 123 (NF-B + DHR), and phagocytosis was measured using the flow cytometer at daily intervals over 6 days in culture.

Figures 5.4-5 shows that differentiated PLB-985 cells displayed increasing levels of phagocytosis of SAPI and latex beads in parallel with increases in the levels of differentiation from day 1 to 5. Incubation with G-CSF or GM-CSF increased the phagocytosis with statistically significant differences ($p \leq 0.05$ or 0.01), observed after day 5 and 6 of culture. In contrast to neutrophils, differentiated PLB-985 cells displayed highest phagocytosis with fluorescent beads, followed by SAPI. However, like neutrophils, levels of phagocytosis of non-fluorescent beads were low. Also, differentiated PLB-985 cells showed low phagocytic ability for SAPI compared to the other particles. Incubation with G-CSF or GM-CSF significantly ($p \leq 0.05$ or 0.01) increased the percentage of positive cells for SAPI and all other particles from day 1-5 as shown in Figures 5.5-7. Representative traces of differentiated PLB-985 cells phagocytosis under these conditions are shown in Figure 5.7.

Forward scatter (FS) versus side scatter (SS) dots plots in Figure 5.8 show that differentiated PLB-985 cells were capable of phagocytosing both the fluorescent and non-fluorescent latex beads, as evident by a population of cells with altered side scatter (SS), indicating increased granularity, and which was enhanced after incubation with the cytokines G-CSF and GM-CSF. In addition, different proportions of the differentiated cells took up between 1 to 6 fluorescent beads as shown in

Figures 5.9-10, which showed that at least 6%, 12%, 5%, 3% and 1% of differentiated PLB-985 cells took up 1, 2, 3, 4, 5 and 6 bead particles, respectively from day 3 to 6 of culture under the various conditions. As for neutrophils, differentiated PLB-985 cells incubated with GM-CSF took up higher numbers of beads per cell, but these differences did not reach statistical significance.

Another important observation revealed by the dot plot traces in Figure 5.8 was that there was increasing number of dead cells from day 4-6 of culture in cells without cytokines (green dots on the left-hand sides of the traces) indicating progression into apoptosis. This accumulation of dead cells was however, less in cells incubated with both G-CSF and GM-CSF, with the latter showing much lower numbers of apoptotic dPLB-985 cells.

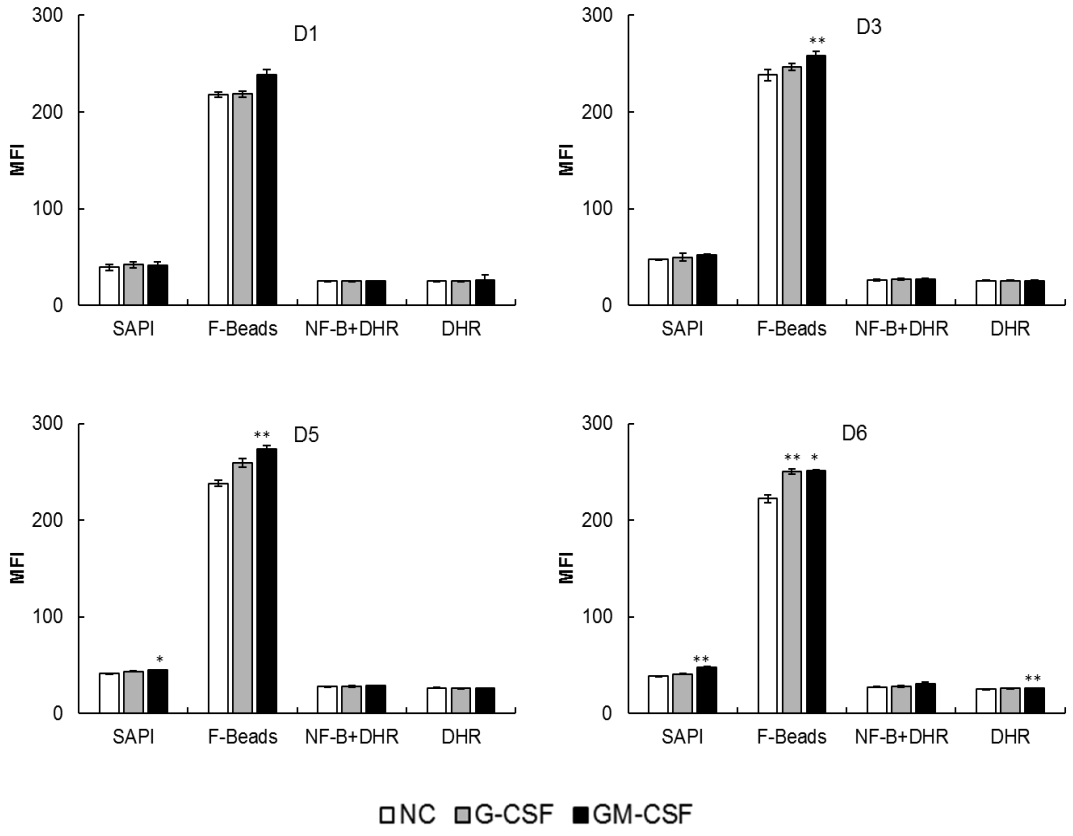


Figure 5.4 Differentiated PLB-985 cells phagocytosis of SAPI and latex beads. dPLB-985 cells were cultured in optimized conditions, involving media changes and cytokine addition for 6 d (see Figure 3.16) and samples were incubated without (NC) and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of SAPI or latex beads at 1:10 (cell to particles ratio), for a further 30 min. DHR 123 (5µM) was added to cells containing non-fluorescent beads (NF-B+DHR) and to the unstimulated cells (DHR only, no particles) for 15 min, and phagocytosis was measured on days 1, 3, 5 and 6 using the flow cytometer. Data are expressed as means of MFI (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

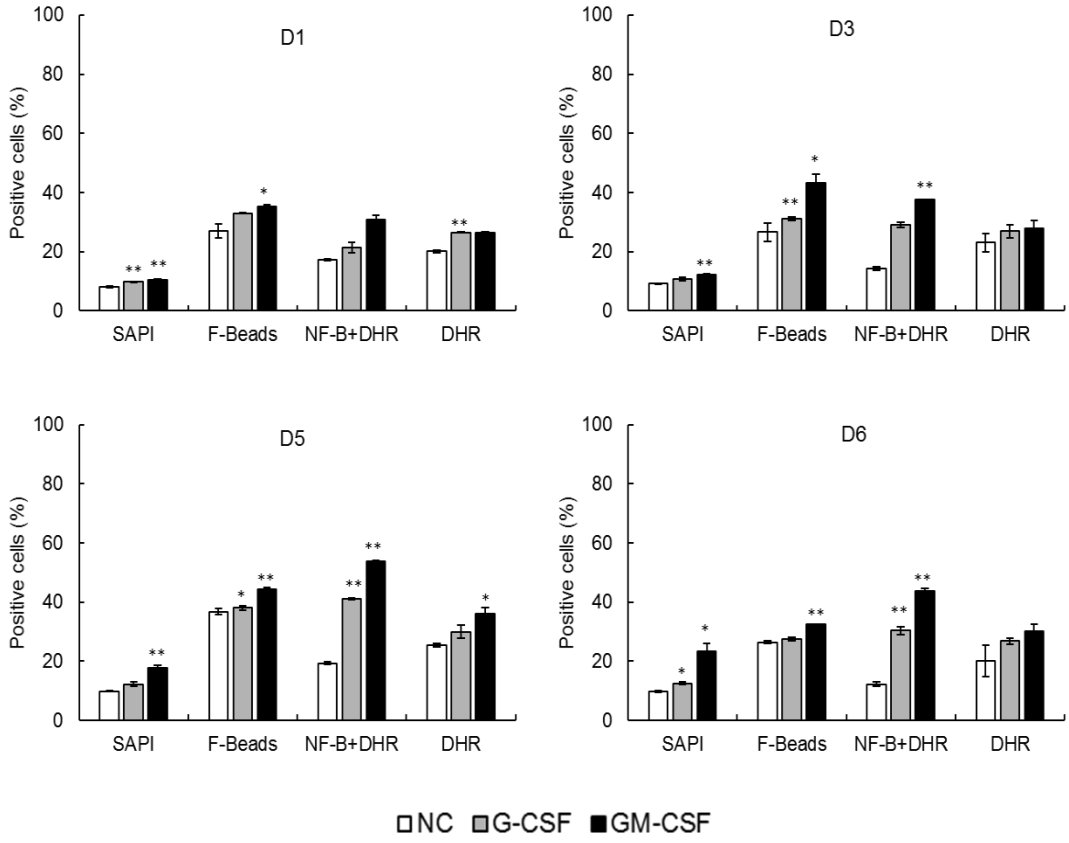


Figure 5.5 Proportions of positive dPLB-985 cells for phagocytosis of SAPI and latex beads. Differentiated PLB-985 cells were cultured in optimized conditions involving media changes and cytokine addition (see Figure 3.16) and samples were incubated without (NC) and with cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of SAPI or latex beads at 1:10 (cell to particles ratio), for a further 30 min. DHR 123 (5µM) was added to cells containing non-fluorescent beads (NF-B+DHR) and to the unstimulated cells (DHR only, no particles) for 15 min, and phagocytosis was measured on days 1, 3, 5 and 6 using the flow cytometer. Similar to neutrophils, treatment with the cytokines significantly increased phagocytosis of both SAPI and latex beads by the differentiated cells. Data are expressed as means of percent positive cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

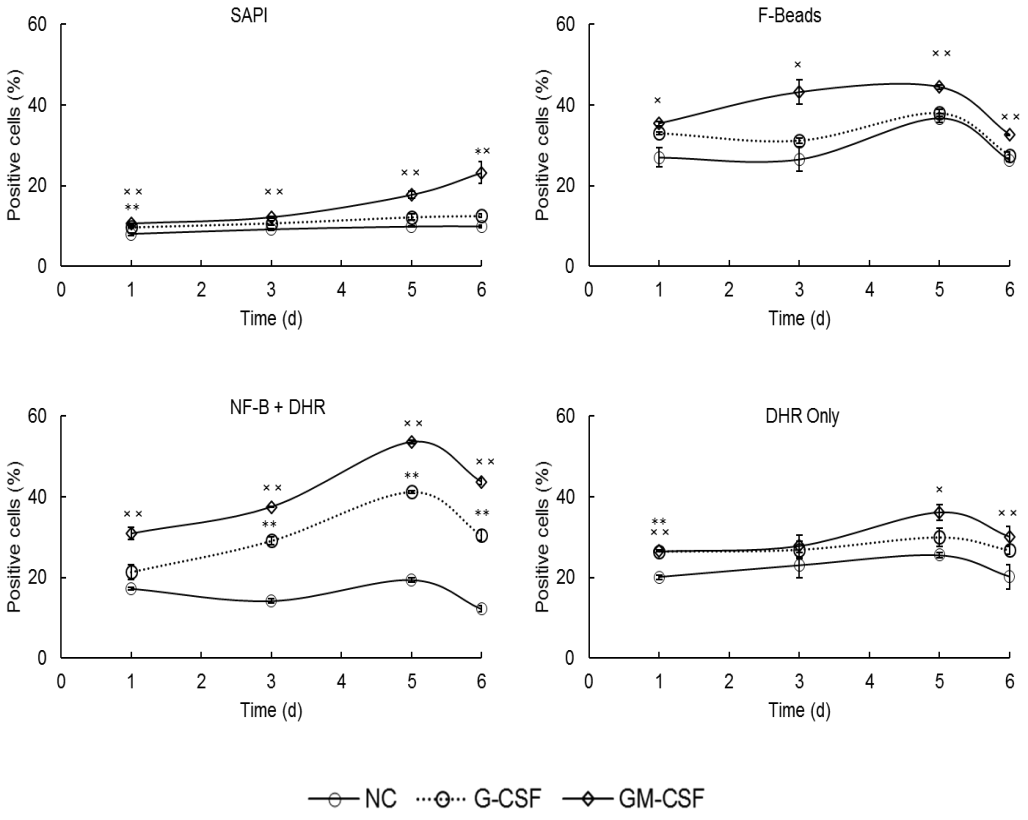
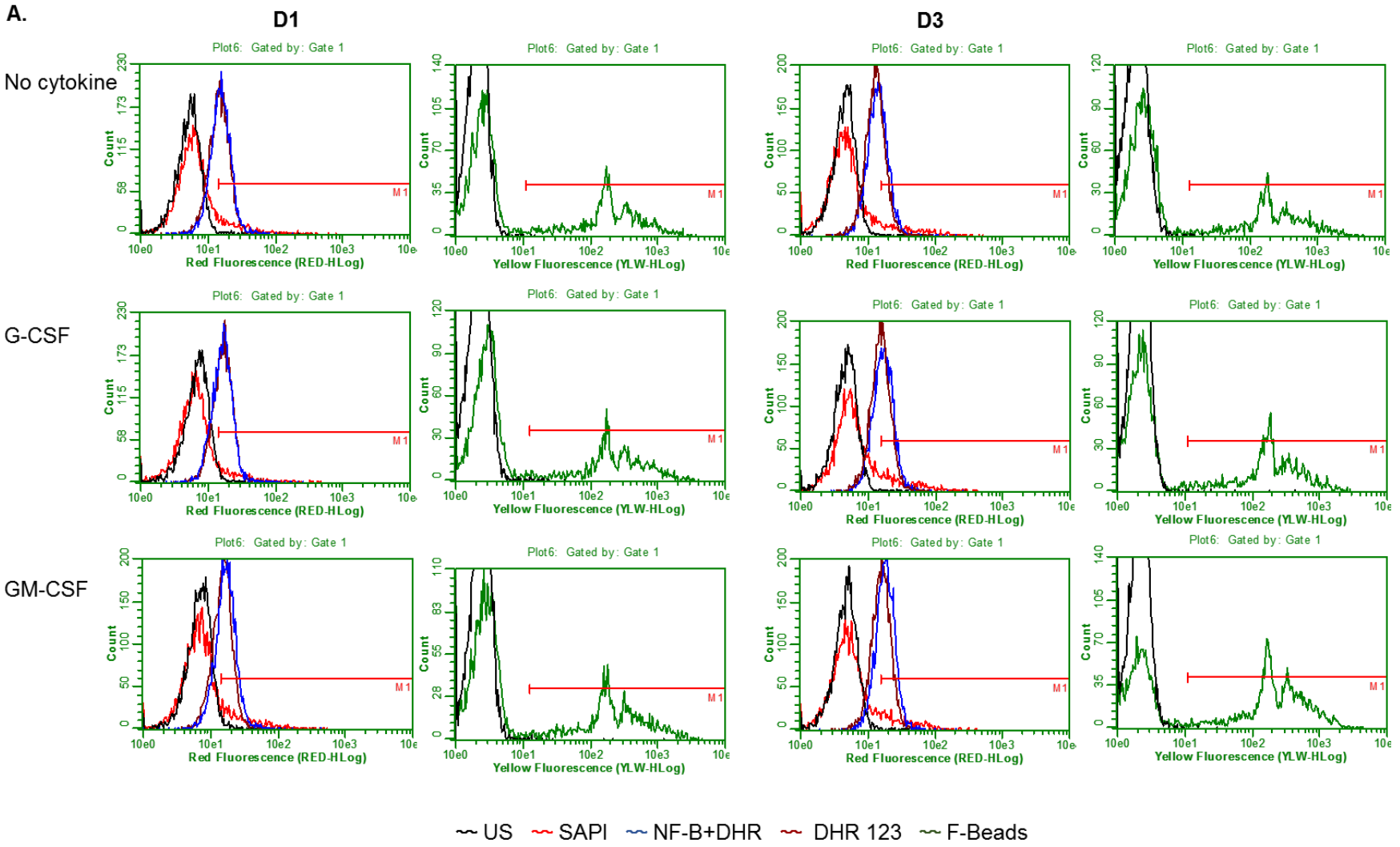


Figure 5.6 Proportions of different particle's phagocytosis by dPLB-985 cells. dPLB-985 cells were cultured in optimized conditions involving media changes and cytokine addition for 6 d (see Figure 3.12) and samples were incubated without (NC) and with cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of SAPI or latex beads at 1:10 (cell to particles ratio), for a further 30 min. DHR 123 (5µM) was added to cells containing non-fluorescent beads (NF-B+DHR) and to unstimulated cells (DHR only, no particles) for 15 min, and phagocytosis was measured on days 1, 3, 5 and 6 using the flow cytometer. Data are expressed as means of percent positive cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test), (* = G-CSF, ˣ = GM-CSF).

Phagocytosis of SAPI and latex beads by dPLB-985 cells



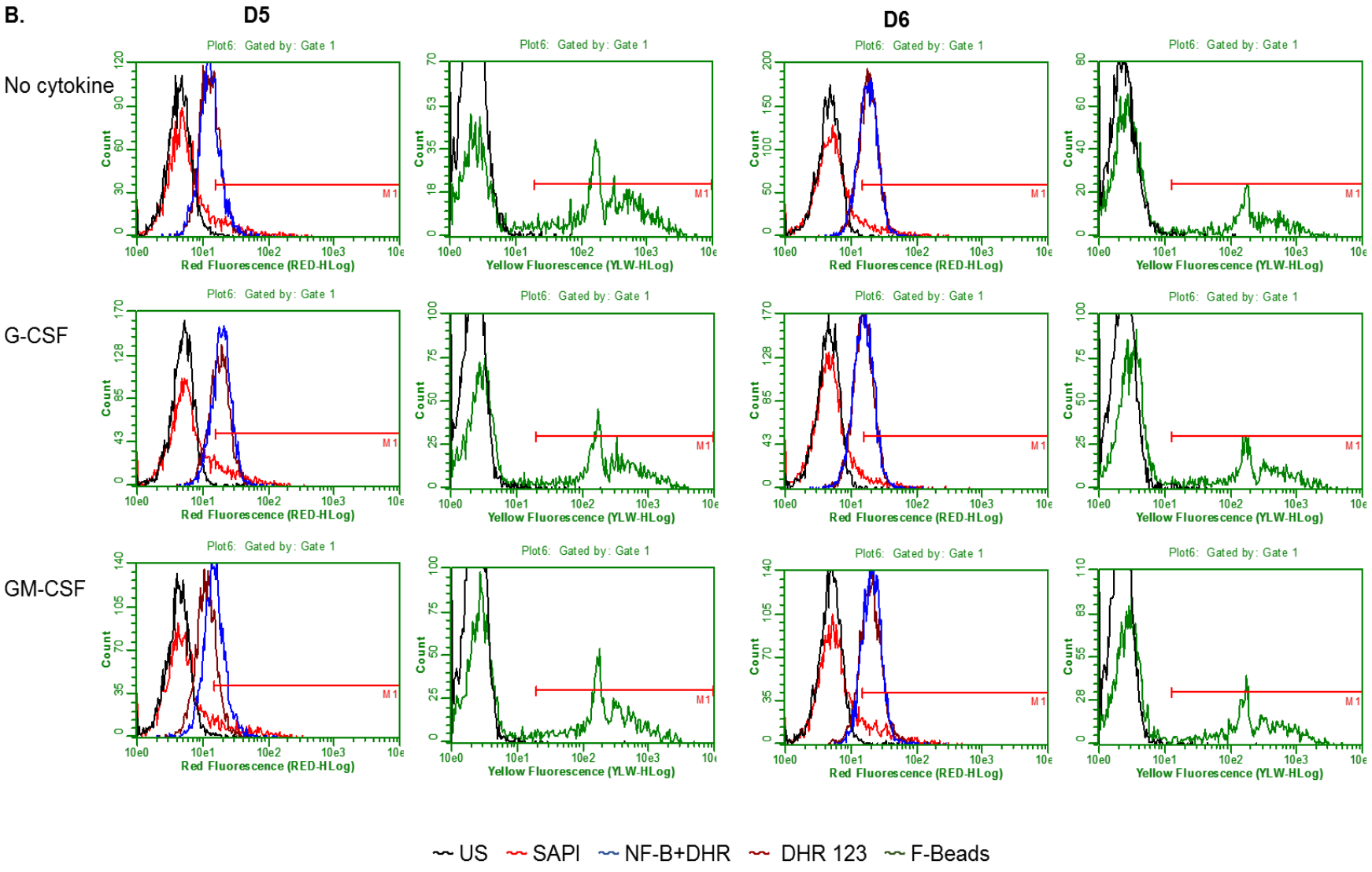
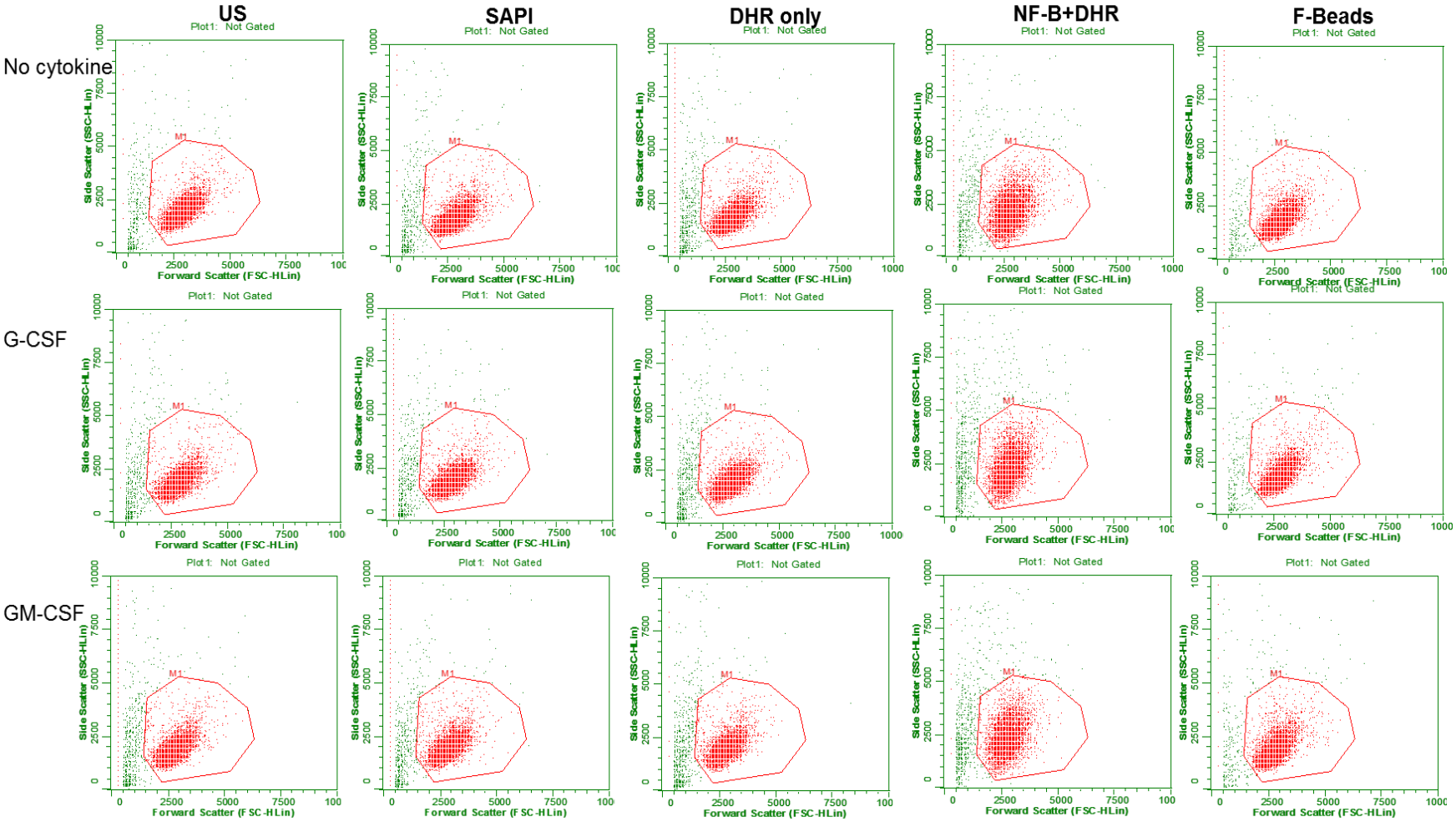
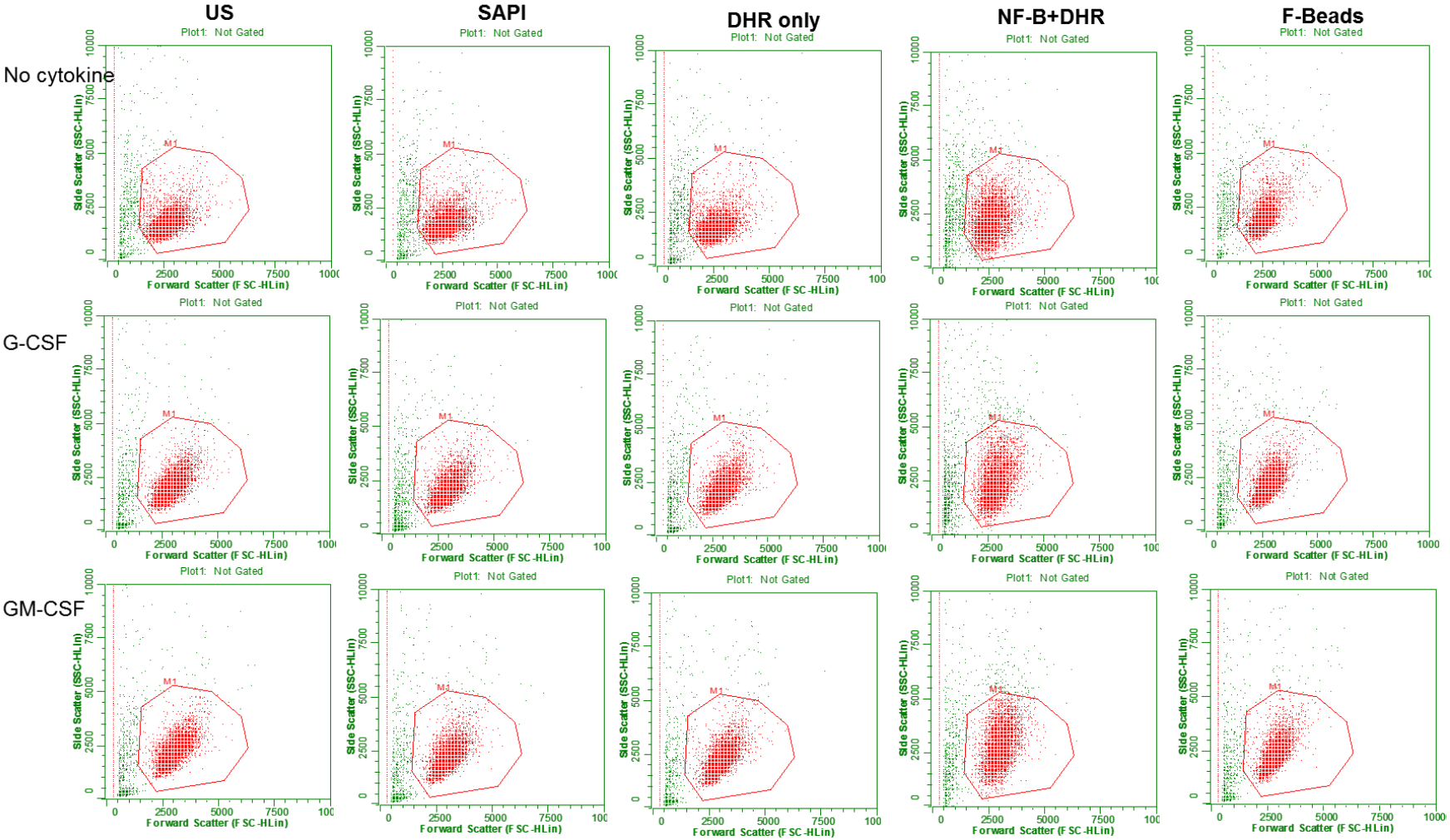

B.
D5
D6
No cytokine
G-CSF
GM-CSF
Plot6: Gated by: Gate 1
Count
Red Fluorescence (RED-HLog)
Yellow Fluorescence (YLW-HLog)
M1
US
SAPI
NF-B+DHR
DHR 123
F-Beads

Figure 5.7 Representative traces of flow cytometer histograms for dPLB-985 cells phagocytosis of SAPI and latex beads. dPLB-985 cells were cultured in optimized conditions, involving media changes and cytokine addition for 6 d as described in Figure 3.16, and samples were incubated without and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of SAPI or latex beads at 1:10 (cell to particles ratio) or unstimulated (US, no particles), for a further 30 min. DHR 123 (5µM) was added to cells containing non-fluorescent beads (NF-B+DHR) and DHR only samples for 15 min. Phagocytosis was measured after days 1, 3, 5 and 6, using the flow cytometer. Unstimulated control (US) cells were used to gate for negative fluorescence. Black traces indicate unstimulated cells. SAPI (red traces) and NF-B + DHR (blue traces) were measured in the red fluorescence channel, while F-Beads (green traces) were read in the yellow fluorescence channel. (A) Days 1&3. (B) Days 5&6.

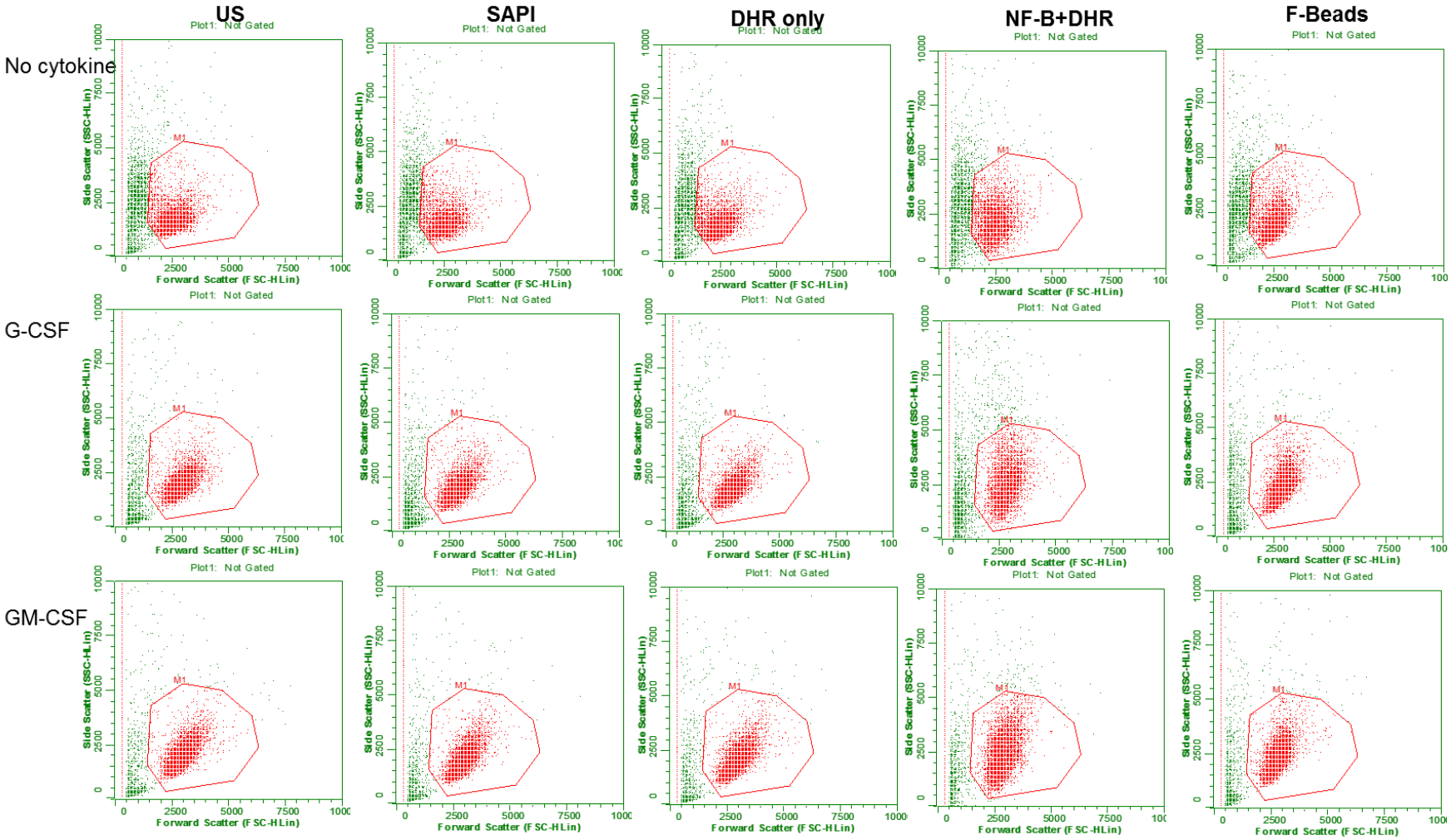
A. Day 1


US
SAPI
DHR only
NF-B+DHR
F-Beads
No cytokine
G-CSF
GM-CSF
Plot1: Not Gated
Side Scatter (SSC-HLin)
Forward Scatter (FSC-HLin)
M1

B. Day 3


US
SAPI
DHR only
NF-B+DHR
F-Beads
No cytokine
G-CSF
GM-CSF
Plot1: Not Gated
Side Scatter (SSC-HLin)
Forward Scatter (F SC-HLin)
M1

C. Day 5


US
SAPI
DHR only
NF-B+DHR
F-Beads
No cytokine
G-CSF
GM-CSF
Plot1: Not Gated
Side Scatter (SSC-HLin)
Forward Scatter (FSC-HLin)
M1

D. Day 6

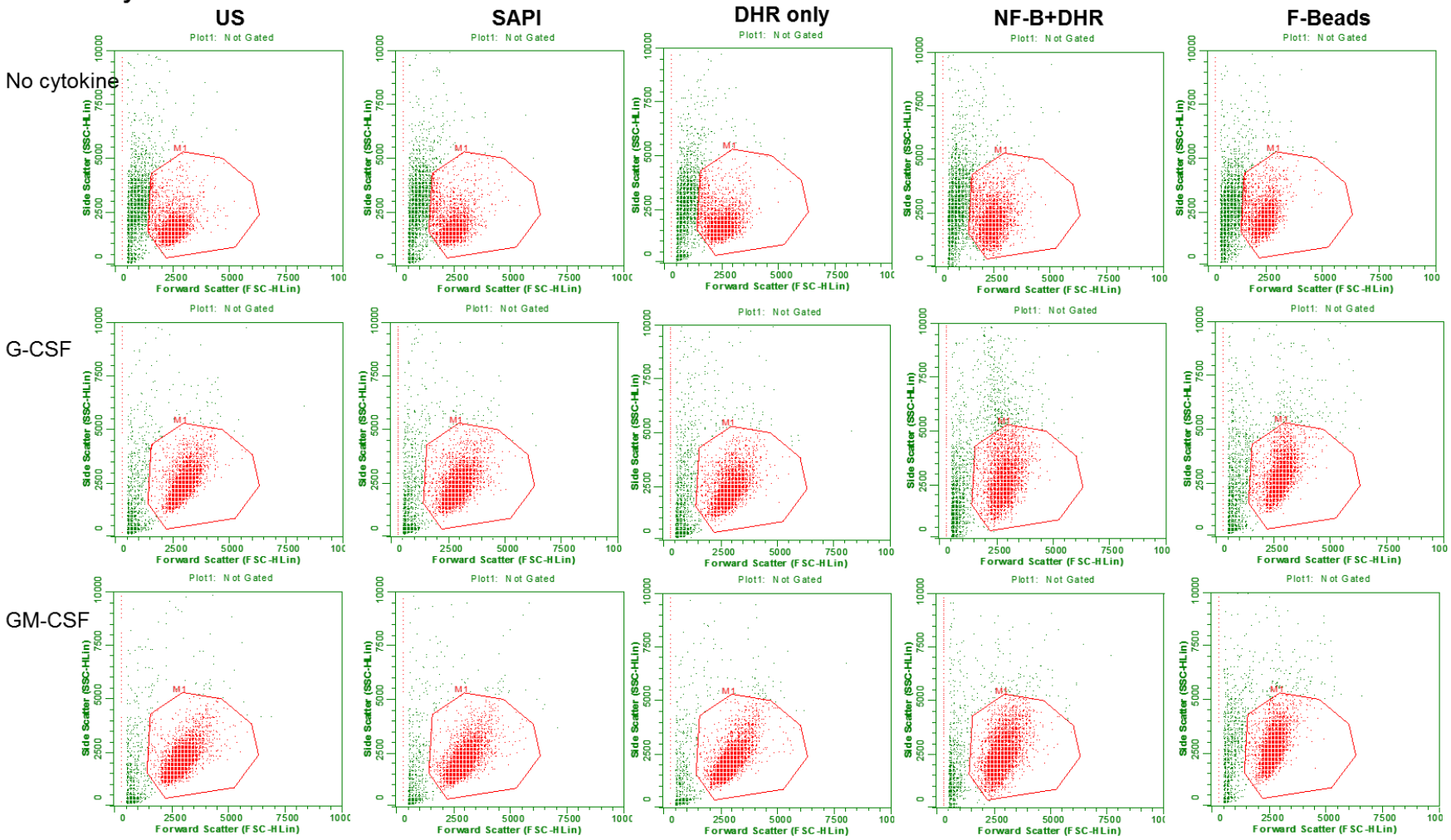

US
SAPI
DHR only
NF-B+DHR
F-Beads
No cytokine
G-CSF
GM-CSF
Plot1: Not Gated
Side Scatter (SSC-HLin)
Forward Scatter (FSC-HLin)
M1

Figure 5.8 Representative traces of flow cytometer dots plots for dPLB-985 cells phagocytosis of SAPI and latex beads. dPLB-985 cells were cultured in optimized conditions, involving media changes and cytokine addition for 6 d (see Figure 3.12) and samples were incubated without and with cytokine G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of SAPI or latex beads at 1:10 (cell to particles ratio) or unstimulated (US, no particles), for a further 30 min. DHR 123 (5µM) was added to cells containing non-fluorescent beads (NF-B+DHR) and DHR only samples for 15 min. Phagocytosis was measured after days 1, 3, 5 and 6, using the flow cytometer. There was apparent altered side scatter shift of cells population in both fluorescent and non-fluorescent beads, indicating increased granularity following beads uptake by the differentiated cells, and this altered SS is higher in the cytokines treated cells. Figures A, B, C, and D for days 1, 3, 5 and 6, respectively.

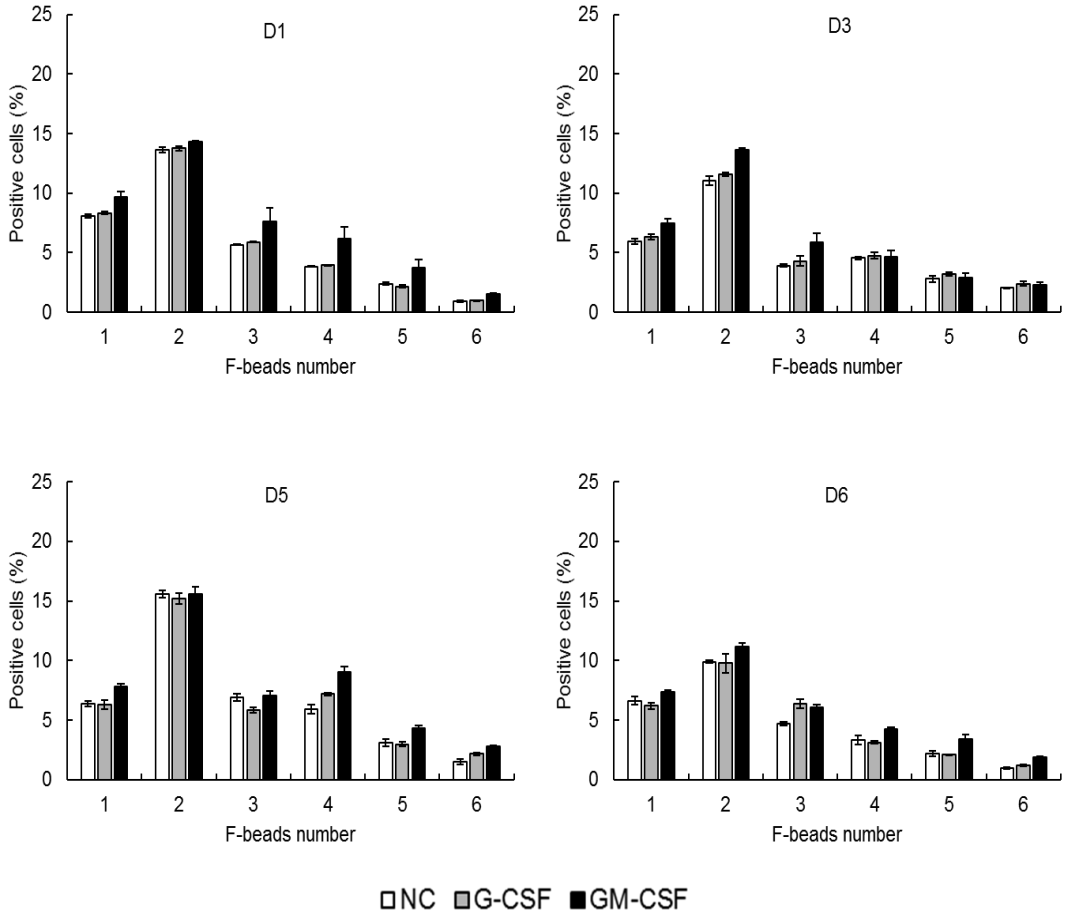


Figure 5.9 Proportions of dPLB-985 cells that phagocytosed different numbers of fluorescent beads. dPLB-985 cells were cultured in optimized conditions, involving media changes and cytokine addition for 6 d (see Figure 3.12) and samples were incubated without (NC) and with cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of fluorescent beads at 1:10 (cell to particles ratio), for a further 30 min, and phagocytosis was measured on days 1, 3, 5 and 6, using the flow cytometer. The percentages of cells that have engulfed I, 2, 3, 4, 5 and 6 beads under the different treatments were calculated from Figure 5.10. Data are expressed as means of percent positive cells (± SEM, n=3).

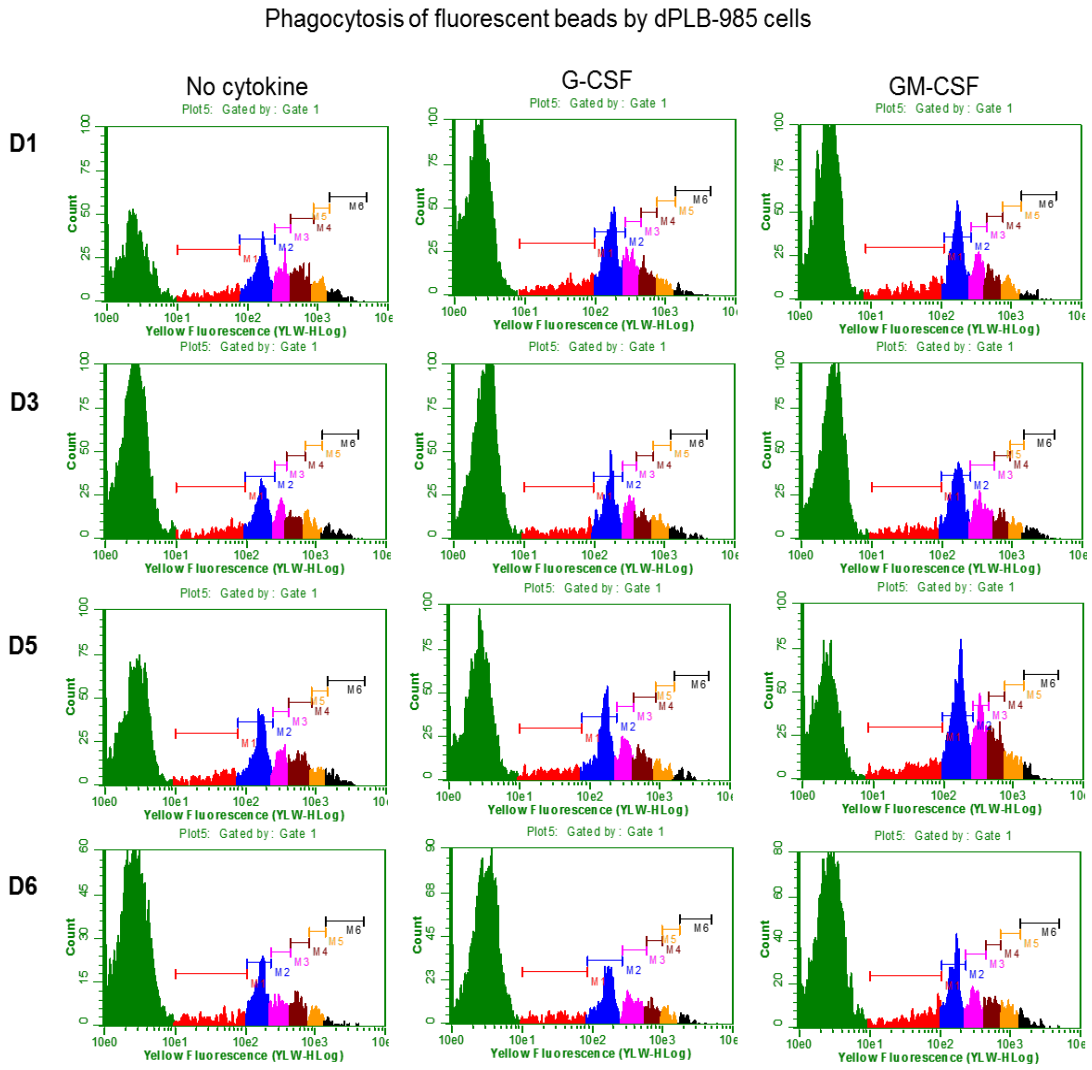


Figure 5.10 Representative flow cytometer traces for proportions of dPLB-985 cells that phagocytosed different numbers of fluorescent beads. dPLB-985 cells were cultured in optimized conditions, involving media changes and cytokine addition for 6 d (described in Figure 3.16) and samples were incubated without and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of fluorescent beads at 1:10 (cell to particles ratio) or unstimulated samples (to gate for negative fluorescence), for a further 30 min, and phagocytosis was measured on days 1, 3, 5 and 6, using the flow cytometer. Green traces indicate cells that did not take up any particle. M1-M6 indicate proportions of cells that have engulfed 1-6 numbers of fluorescent beads, under the various conditions.

5.3.3 Neutrophils oxidative burst activity by flow cytometry

Phagosome formation following engulfment of particles by neutrophils triggers oxidative burst activity to generate ROS within the phagosome for bacterial killing. It was therefore necessary to measure the oxidative burst generation of isolated neutrophils and differentiated PLB-985 cells after phagocytosis. Freshly-isolated neutrophils, incubated without and with G-CSF (10ng/mL) or GM-CSF(5ng/mL), were stimulated for the oxidative burst by addition of fMLP (1µM), PMA (0.1µg/mL) or latex beads at 10:1 (particles to cell ratio). ROS generation was then measured by DHR 123 fluorescence, using the flow cytometer.

Figure 5.11A shows the fluorescence measurements of neutrophil oxidative burst activity. Neutrophils without cytokine (NC) stimulated by PMA displayed high activity (MFI 352.64 ± 1.85) which significantly ($p \leq 0.05$, or 0.01) increased following incubation with cytokines G-CSF (MFI 548.80 ± 14.13) and GM-CSF (MFI 627.32 ± 24.18). The fMLP produced low activity in cells with no cytokine (NC) (MFI 39.77 ± 0.98), which significantly ($p \leq 0.01$) increased by incubation with G-CSF (MFI 41.44 ± 0.51) and GM-CSF (MFI 89.23 ± 4.43). Non-fluorescent beads also exhibited lower oxidative burst activity (MFI 30.43 ± 0.79) which increased slightly following incubation with G-CSF (MFI 32.41 ± 0.52) and significantly ($p \leq 0.05$) with GM-CSF (MFI 44.38 ± 2.40). DHR 123 only (no particles) cells showed the least ROS generation (MFI 26.65 ± 0.22), which increased insignificantly after incubation with G-CSF (MFI 28.08 ± 1.55) and significantly ($p \leq 0.01$) with GM-CSF (MFI 29.75 ± 0.58). The fluorescence produced by DHR only cells was likely generated from mitochondrial oxidation, and the increased fluorescence observed in latex beads with DHR indicates that the beads have been taken up by neutrophils and have triggered ROS production.

The percent positive cells shown in Figure 5.11B followed similar patterns. However, the percentages of positive cells were very high with PMA (>98%), fMLP (>73%), latex beads (>71%) under the different conditions, while DHR only cells showed very low percentage of positive cells (23.42% - 42.77%). Representative flow cytometer traces of neutrophil oxidative burst activity, under these conditions is shown in Figure 5.12.

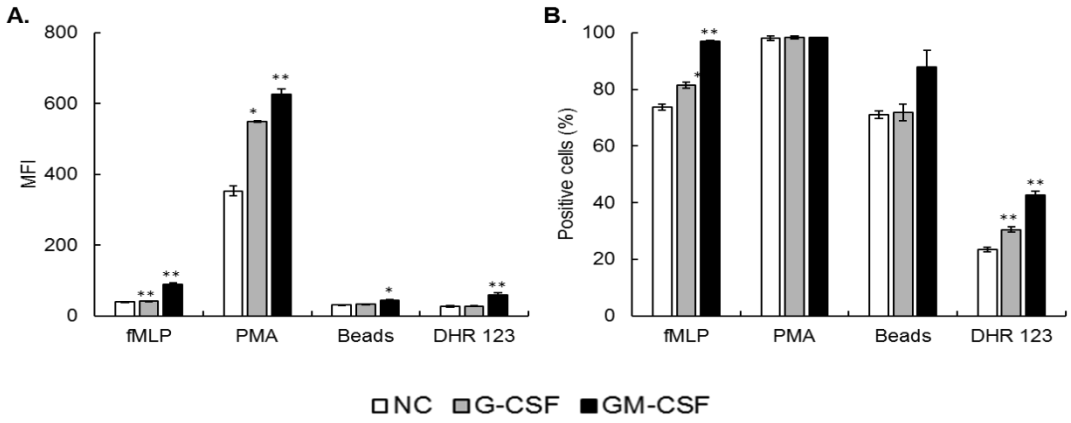


Figure 5.11 Neutrophil oxidative burst activity by flow cytometry. Freshly-isolated blood neutrophils were incubated without (NC) and with cytokine G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by DHR 123 (5µM) for 15 min and oxidative burst stimulated by addition of fMLP (1µM), PMA (0.1µg/mL), non-fluorescent latex beads at 1:10 (cells to particles ratio) or unstimulated samples (to gate for negative fluorescence), for a further 15 min. ROS generation was then measured using the flow cytometer. (A) Mean fluorescence intensity (MFI) of neutrophils oxidative burst. (B) Percentage of positive cells for oxidative burst activity calculated from Figure 12. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

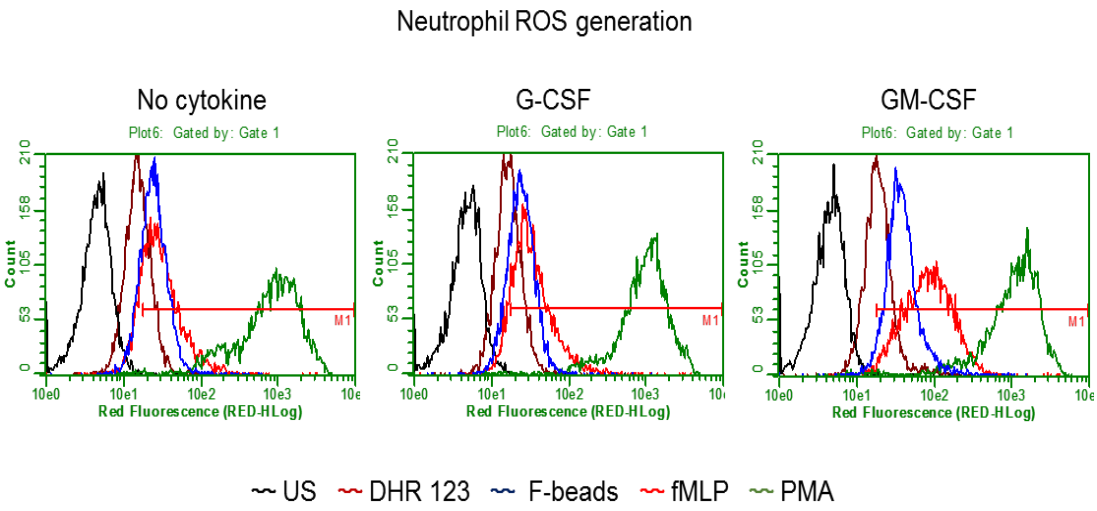


Figure 5.12 Representative traces of flow cytometer histogram for neutrophil oxidative burst activity. Freshly-isolated neutrophils were incubated without and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by DHR 123 (5µM) for 15 min and oxidative burst stimulated by addition of fMLP (1µM), PMA (0.1µg/mL), non-fluorescent latex beads at 1:10 (cells to particles ratio) or unstimulated (US) to gate for negative fluorescence, for a further 15 min. ROS generation was then measured using the flow cytometer. Black traces indicate unstimulated control cells. All traces were measured in the red fluorescence channel.

5.3.4 dPLB-985 cells oxidative burst activity by flow cytometry

Non-differentiated PLB-985 and dPLB-985 cells differentiated under the optimised conditions, involving media changes and cytokine addition as described in Figure 3.16, were incubated without and with G-CSF (10ng/mL) or GM-CSF (5ng/mL), and then stimulated for oxidative burst activity by addition of fMLP (1µM), PMA (0.1µg/mL), latex beads at 1:10 (cell to particles ratio) or unstimulated (US, for negative fluorescence), in cultures containing DHR 123 (5µM). ROS generation was then measured using the flow cytometer.

Non-differentiated PLB-985 cells did not produce oxidative burst activity, under any of these conditions (data not shown). However, following differentiation into neutrophil-like cells, these cells displayed increasing ROS generation parallel with the levels of differentiation from day 1 to 6 as shown in Figure 5.13. Like the isolated neutrophils, differentiated PLB-985 cells stimulated with PMA showed high oxidative burst activity which increased significantly ($p \leq 0.05$, or 0.01) after incubation with both G-CSF and GM-CSF from day 1-6 of culture. However, contrary to neutrophils, both fMLP and latex beads induced only slight and insignificant oxidative burst activity in the differentiated PLB-985 cells under these conditions. The fMLP response in neutrophils is mediated by formyl peptide receptors (Hallett & Lloyds, 1995), which might not be expressed on the surface of differentiated PLB-985 cells. The latex beads were also unable to trigger oxidative burst activity, even though they can be internalised by the differentiated PLB-985 cells as shown in Figures 5.5-7.

Figure 5.14 shows the percentage of positive differentiated PLB-985 cells for oxidative burst activity which revealed the same patterns with the fluorescence intensities described in Figure 5.13, but in contrast to the MFI data, after incubation with G-CSF or GM-CSF, fMLP and latex beads showed significant ($p \leq 0.05$, or 0.01)

increase in percent positive cells from day 3-5. In cultures without cytokine (NC), the PMA response showed biphasic change on days 3-5 (high and low responders) and then declined to low responders on day 6. However, incubation with both G-CSF and GM-CSF maintained high responders for longer times. Representative traces of differentiated PLB-985 cells oxidative burst activity under these conditions is shown in Figure 5.15.

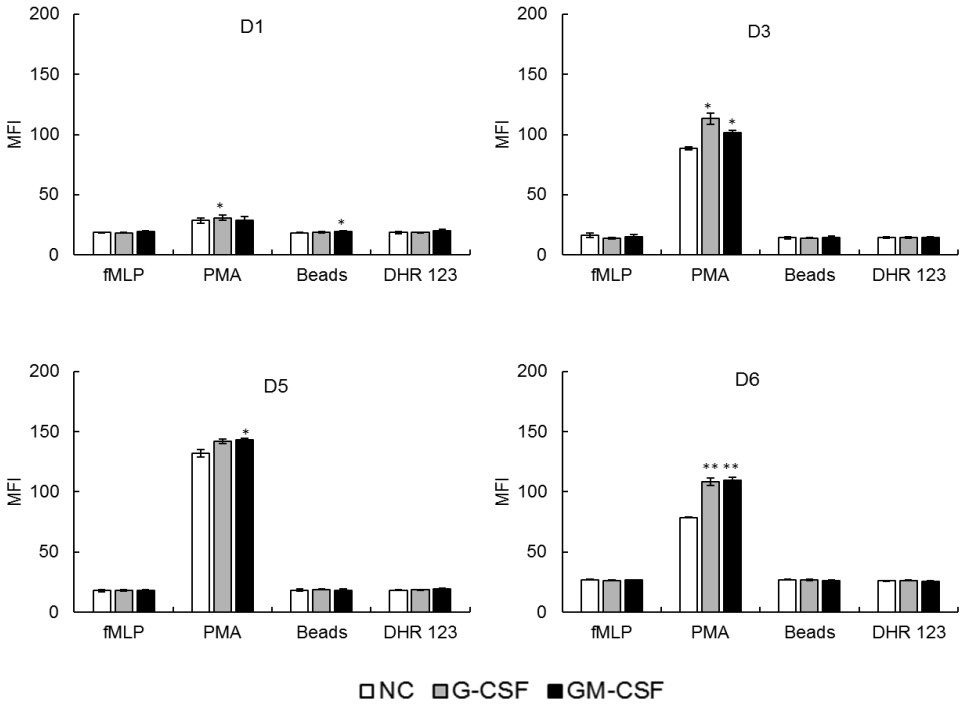


Figure 5.13 Differentiated PLB-985 cells oxidative burst activity by flow cytometry. dPLB-985 cells were cultured in optimized conditions, involving media changes and cytokine addition for 6 d (see Figure 3.16) and samples were incubated without (NC) and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of DHR 123 (5µM). The oxidative burst activity was then stimulated by addition of fMLP (1µM), PMA (0.1µg/mL), non-fluorescent latex beads at 1:10 (cell to particles ratio) or unstimulated (for negative fluorescence), for a further 15 min, and ROS generation was measured using the flow cytometer. Data are expressed as means of MFI (± SEM, n=4), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

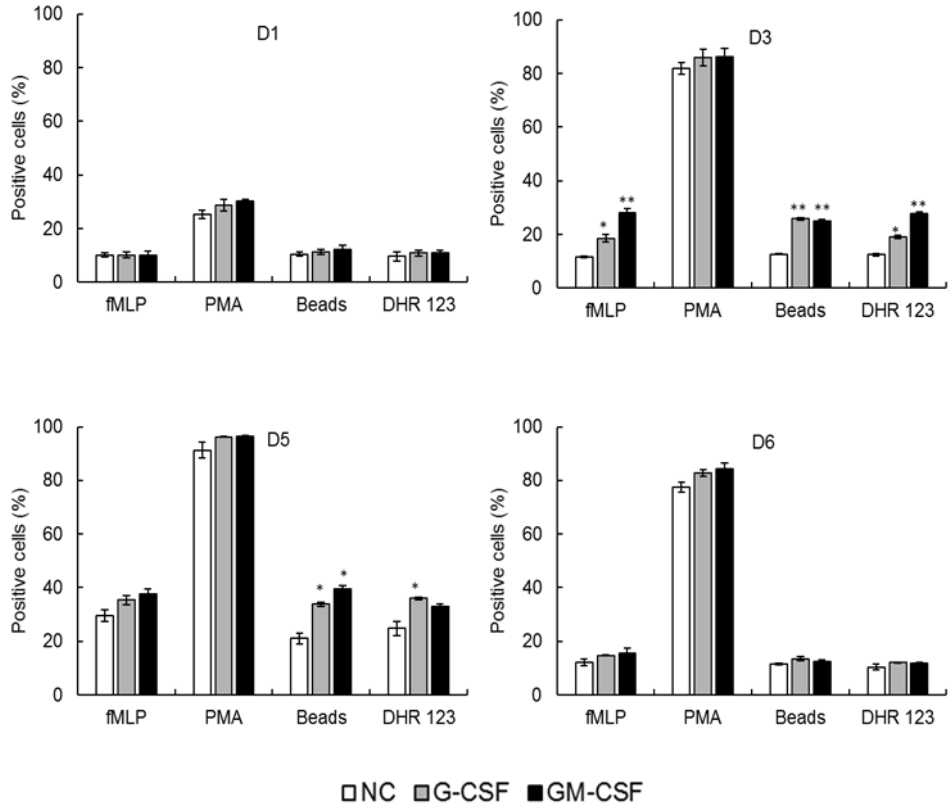


Figure 5.14 Percentage of positive dPLB-985 cells for oxidative burst activity by flow cytometry. dPLB-985 cells were cultured in optimized conditions, involving media changes and cytokine addition for 6 d (described in Figure 3.16) and samples were incubated without (NC) and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of DHR 123 (5µM) for further 15 min. The oxidative burst activity was then stimulated by addition of fMLP (1µM), PMA (0.1µg/mL), non-fluorescent latex beads at 1:10 (cell to particles ratio) or unstimulated (for negative fluorescence) for a further 15 min, and ROS generation was measured using the flow cytometer. Data are expressed as means of percent total cells (± SEM, n=4), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

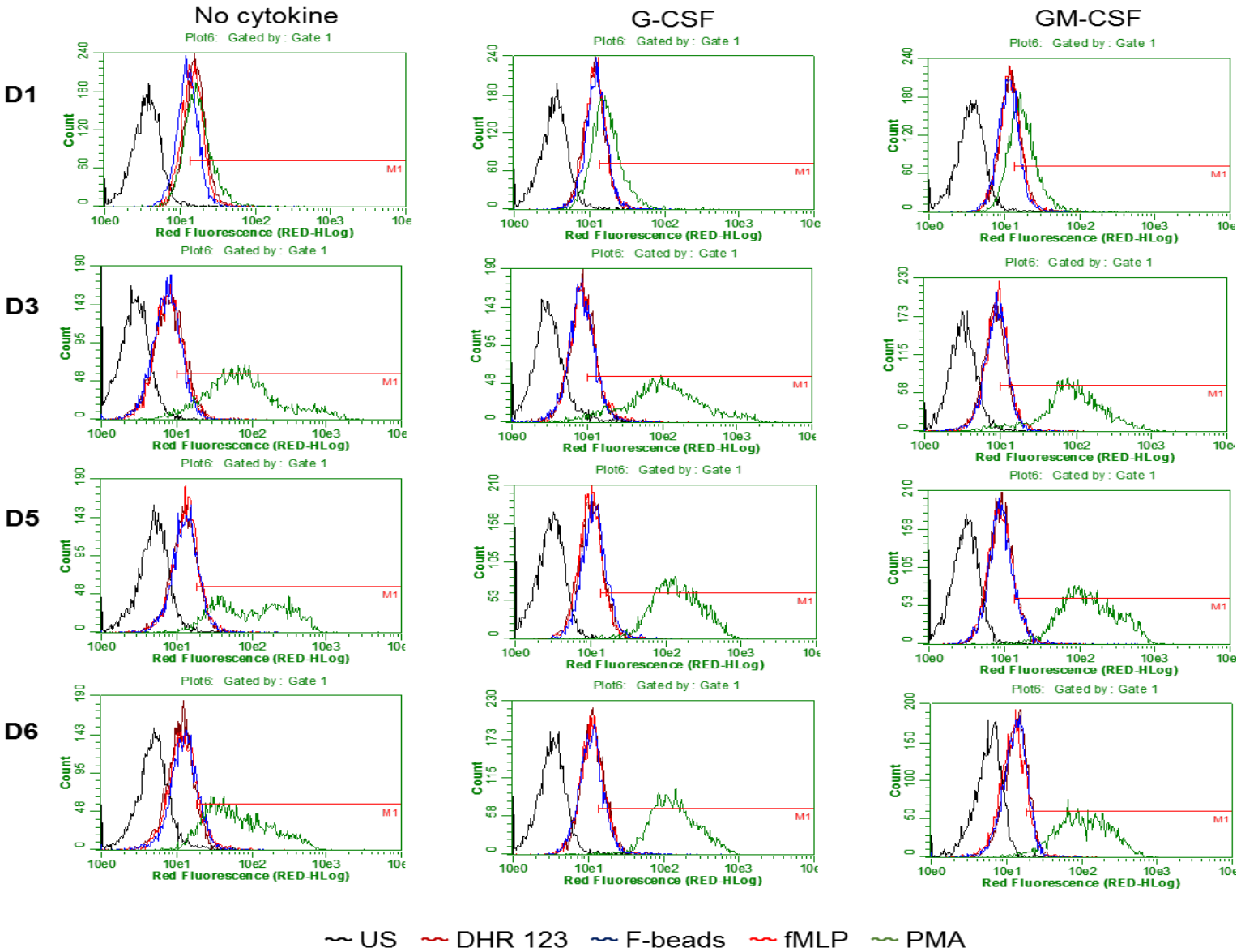

No cytokine
G-CSF
GM-CSF
D1
D3
D5
D6
Plot6: Gated by : Gate 1
Count
Red Fluorescence (RED-HLog)
M1
US
DHR 123
F-beads
fMLP
PMA

Figure 5.15 Representative traces of flow cytometer histograms for dPLB-985 cells oxidative burst activity. dPLB-985 cells were cultured in optimized conditions, involving media changes and cytokine addition for 6 d, as described in Figure 3.16 and samples were incubated without and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, followed by addition of DHR 123 (5µM) for 15 min. The oxidative burst activity was then stimulated by addition of fMLP (1µM), PMA (0.1µg/mL), non-fluorescent latex beads at 1:10 (cell to particles ratio) or unstimulated (to gate for negative fluorescence) for a further 15 min, and ROS generation was measured using the flow cytometer. Black traces indicate unstimulated (control) cells. All traces were measured in the red fluorescence channel. In cultures without cytokines (NC), the PMA response showed a biphasic change on days 3-5 (high and low responders) and then declined to low responders on day 6, whilst incubation with both G-CSF and GM-CSF maintained high responders for longer time. (A) Day 1-3. (B) Day 3-6.

5.3.5 Neutrophils oxidative burst activity by chemiluminescence assay

To measure the total (intra- and extra-cellular) ROS generation by neutrophils and differentiated PLB-985 cells following phagocytosis, luminol enhanced luminescence assay was used as luminol can pass through the cell membrane and become oxidised by ROS, releasing energy in the form of light that gives a measure of total oxidant generation. Freshly-isolated neutrophils were cultured without and with GM-CSF (5ng/mL), and then oxidative burst activity was stimulated with fMLP (1µM) or PMA (0.1µg/mL), or un-stimulated (control) samples, and the total ROS generation was measured by the luminol-enhanced chemiluminescence assay using a luminometer.

As shown in Figure 5.16, neutrophils with no cytokine (NC) stimulated by PMA produced a very high chemiluminescence response (RLU 468.01 ± 28.15) followed by cells stimulated by fMLP (RLU 112.03 ± 15.64) while un-stimulated cells (US) showed the lowest response (RLU 24.74 ± 7.28). Strikingly, incubation with GM-CSF significantly ($p \leq 0.01$ or 0.001) increased responses in fMLP stimulated cells (RLU 534.97 ± 20.99) and un-stimulated cells (RLU 81.77 ± 3.47), but there was only a slight and insignificant increase in PMA stimulated cells (RLU 493.17 ± 36.24). PMA is membrane permeable and does not require priming to activate neutrophils. In contrast, fMLP binds formyl peptide receptors (FPR) on the granules and therefore, requires priming of neutrophils to mobilise the granules to the cell surface, to stimulate an oxidative burst (Hallett & Lloyds, 1995). Representative traces for oxidative burst activity of neutrophils under these conditions are shown in Figure 5.17.

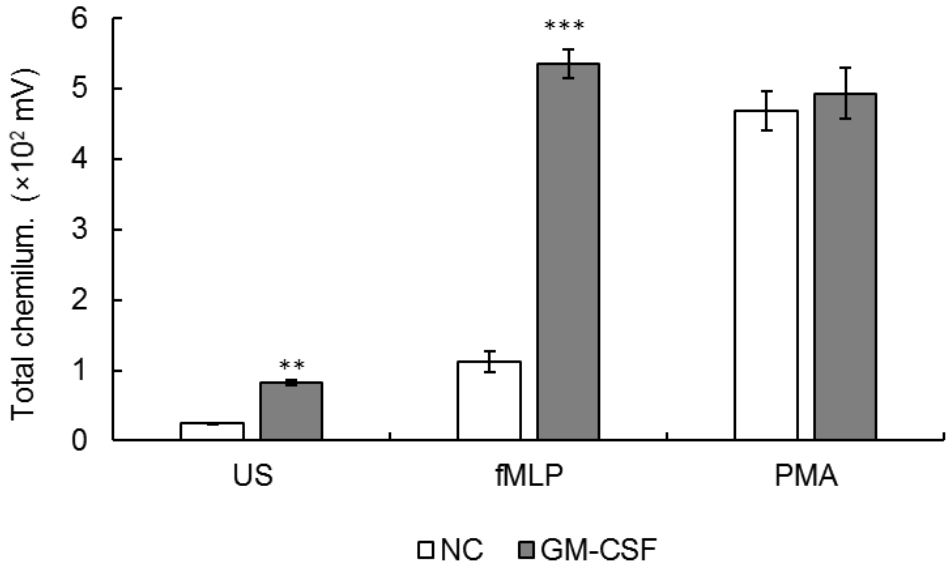


Figure 5.16 Total chemiluminescence for neutrophil oxidative burst activity. Freshly-isolated blood neutrophils were incubated without (NC) and with the cytokine GM-CSF (5ng/mL) for 45 min. The oxidative burst activity was then stimulated by addition of fMLP (1µM), PMA (0.1µg/mL) or un-stimulated (US). Luminol-enhanced chemiluminescence assay was used to measure ROS generation, using a luminometer. Data are expressed as means of total chemiluminescence (± SEM, n=4, ** = p≤0.01, *** = p≤0.001 (paired, two-tailed student's t-test).

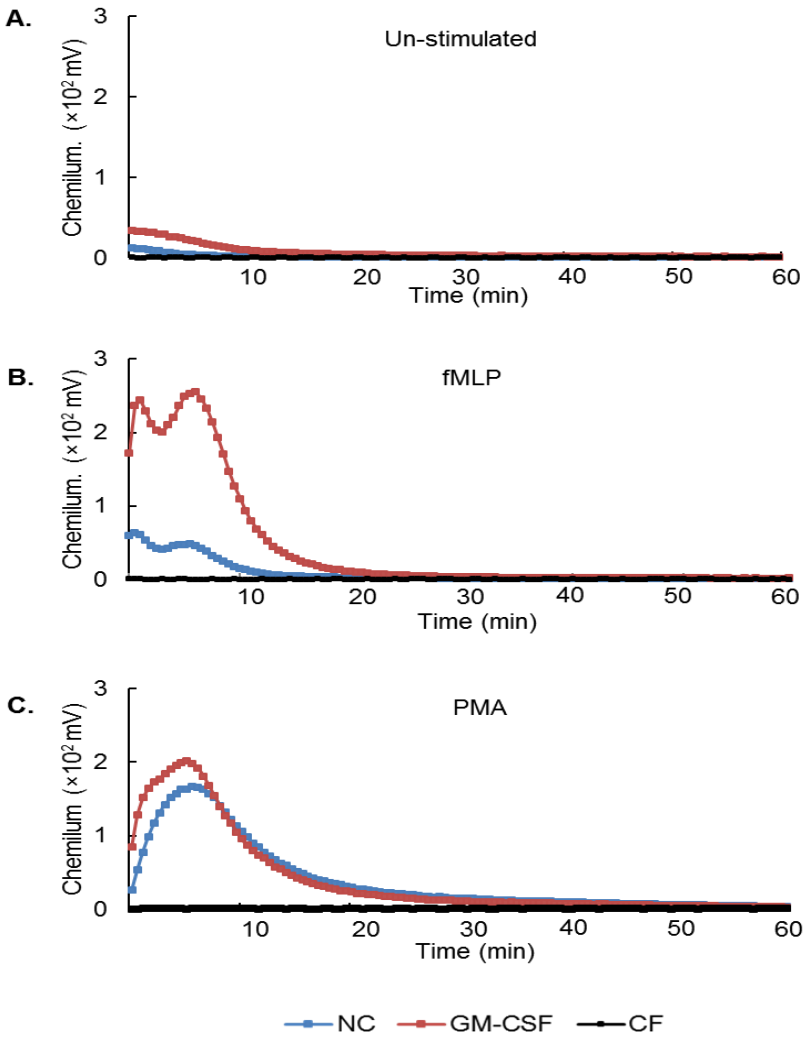


Figure 5.17 Representative chemiluminescence traces for neutrophil oxidative burst activity. Freshly-isolated blood neutrophils were incubated without (NC) and with the cytokine GM-CSF (5ng/mL) for 45 min. The oxidative burst was then stimulated by addition of fMLP (1µM) or PMA (0.1µg/mL) including un-stimulated (US) and cell-free (CF) samples (for negative chemiluminescence). The luminol-enhanced chemiluminescence assay was used to measure ROS generation using a luminometer. The fMLP induces a biphasic chemiluminescence response peaks in neutrophils with the initial peak within 2-3 min and the second peak at within 5-7 min after addition of stimulus, indicating extra-and intra-cellular oxidants generation, respectively. In contrast, PMA induces a single response peak reaching maximum at around 5-7 min, indicating intracellular oxidant generation.

5.3.6 dPLB-985 cells oxidative burst activity by chemiluminescence assay

Non-differentiated PLB-985 and dPLB-985 cells differentiated under the optimised conditions, involving media changes and cytokine addition as described in Figure 3.16, were incubated without and with the cytokine GM-CSF (5ng/mL) for 45 min. The oxidative burst activity was then stimulated by addition of fMLP (1µM), PMA (0.1µg/mL), or un-stimulated (US). Total ROS generation was measured by luminol-enhanced chemiluminescence assay using a luminometer.

Figure 5.18 shows the total ROS produced by non-differentiated PLB-985 (A) and dPLB-985 cells (at day 5 in culture) (B). Non-differentiated PLB-985 cells produced insignificant total ROS following stimulation with both fMLP and PMA, and which did not change significantly, after incubation with the cytokine GM-CSF. This shows that non-differentiated PLB-985 cells are incapable of ROS generation. Interestingly, differentiated PLB-985 cells showed high PMA stimulated total ROS (RLU 148.37 ± 23.93) which increased slightly but insignificantly, after incubation with GM-CSF (RLU 160.88 ± 9.09). This is not unexpected, as PMA response is receptor-independent and it activates ROS production by directly stimulating protein kinase C. The fMLP stimulated differentiated PLB-985 cells showed insignificant total ROS generation (RLU 57.03 ± 2.11), which increased slightly but insignificantly after incubation with GM-CSF (RLU 59.87 ± 12.78). Like blood neutrophils, differentiated PLB-985 cells showed oxidative burst activity in response to PMA stimulation. However, in contrast to neutrophils, these cells could not be stimulated for ROS production by fMLP (which activates the receptor-coupled oxidative burst). These observations are consistent with the flow cytometry data of ROS generation by differentiated PLB-985 cells, described in section 5.3.4. Representative traces of oxidative burst activity of PLB-985 and dPLB-985 cells under these conditions is shown in Figure 5.19.

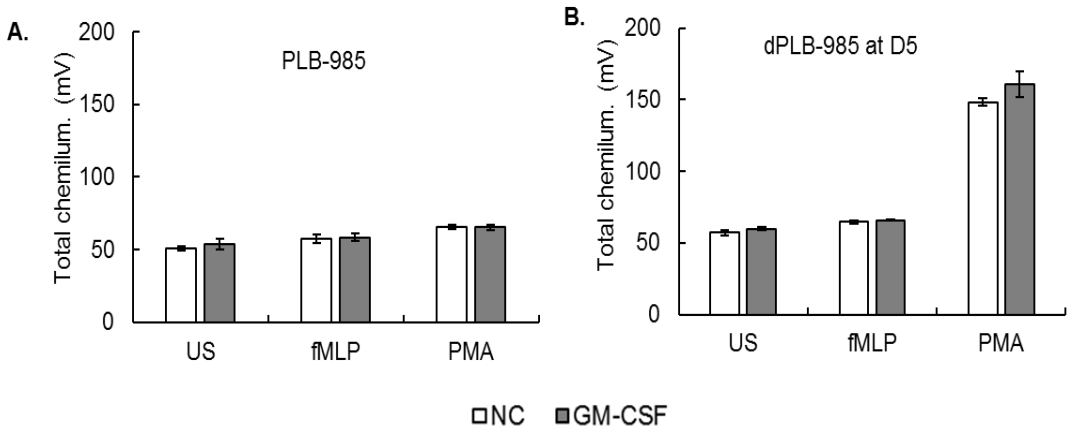


Figure 5.18 Total chemiluminescence for PLB-985 cells oxidative burst activity. Non-differentiated PLB-985 and dPLB-985 cells (day 5) differentiated under the optimised conditions, involving media changes and cytokine addition (see Figure 3.16), were incubated without (NC) and with the cytokine GM-CSF (5ng/mL) for 30 min. The oxidative burst was then stimulated by addition of fMLP (1µM), PMA (0.1µg/mL), or un-stimulated (US) samples. Luminol-enhanced chemiluminescence assay was used to measure ROS generation using a luminometer. (A) Total ROS generated by non-differentiated PLB-985 cells. (B) Total ROS generated by dPLB-985 cells. Data are expressed as means of total chemiluminescence (± SEM, n=4).

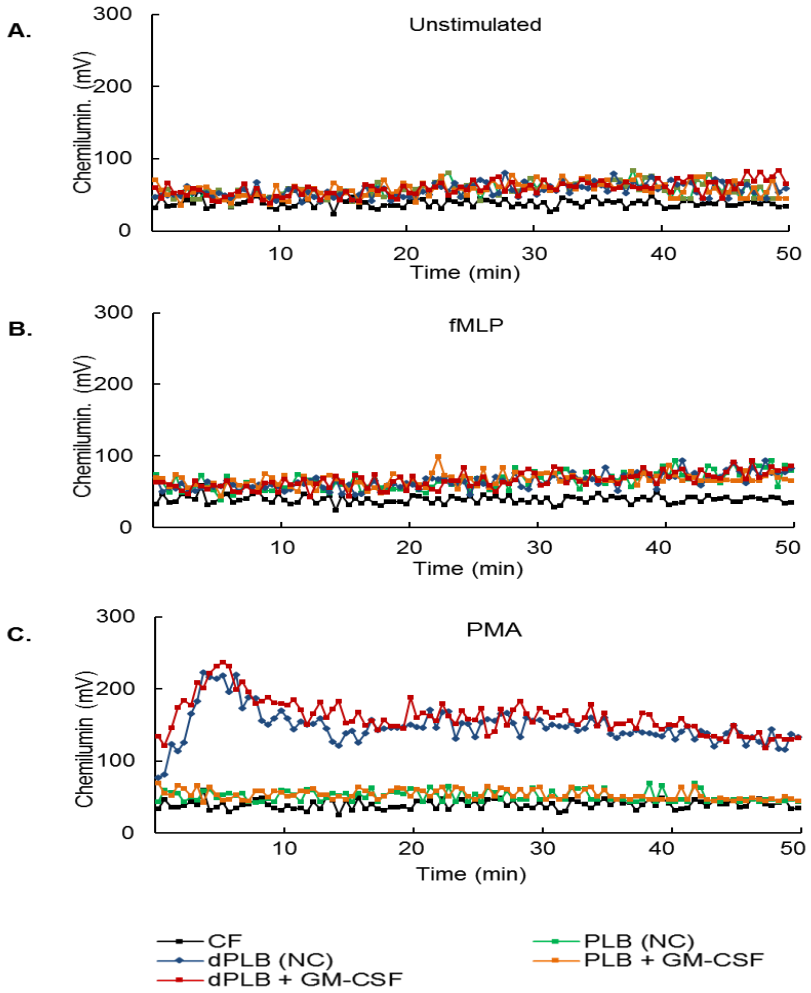


Figure 5.19 Representative chemiluminescence assay traces for dPLB-985 cells oxidative burst activity. PLB-985 and dPLB-985 cells (day 5) differentiated under the optimised conditions as described in Figure 3.16, were incubated without (NC) and with the cytokine GM-CSF (5ng/mL) for 30 min. The oxidative burst activity was stimulated by addition of fMLP (1µM) or PMA (0.1µg/mL), including unstimulated (US) and cells-free (CF) samples (for negative chemiluminescence). The luminol-enhanced chemiluminescence assay was used to measure ROS generation using a luminometer. PMA induced a single response peak in dPLB-985 cells, but no activity was detected with fMLP stimulation in these cells. The non-differentiated PLB-985 cells did not produce chemiluminescence in response to either PMA or fMLP stimulation.

5.4 Discussion and conclusions

Phagocytic cells of the immune system protect the host against microbial infection by their ability to recognise, engulf and eliminate invading pathogens and cell debris, through complex and tightly-regulated mechanisms of phagocytosis and oxidative burst (Pivot-Pajot et al., 2010, Martin & Bhakdi, 1991). Landmark work by Baldridge and Gerard revealed a marked association between the processes of phagocytosis and the generation of the oxidative burst during pathogen killing, when they demonstrated that phagocytosis of bacteria was accompanied by rapid burst of oxygen uptake (Gerard & Baldrige, 1933). Allen *et al*. also observed an emission of a burst of chemiluminescence by neutrophils during phagocytosis (Allen, et al., 1972). The primary product of respiratory burst was later confirmed to be the superoxide anion (O_2^-) derived from NADPH oxidase system and that it was responsible for initiation of the bacterial killing (Babior, et al., 1973).

Neutrophils possess many cytotoxic components and associated pathways, such as variety of cytotoxic enzymes (e.g., hydrolases, nucleases and proteases) in their granules plus the ability to generate reactive oxygen metabolites via non-mitochondrial O_2 uptake during an oxidative burst, from activated NADPH oxidase and myeloperoxidase systems. The reactive oxygen metabolites play a crucial role in pathogen killing and are perhaps involved in destroying some types of tumours (Edwards et al., 1989). These properties have made neutrophils the most adapted for microbial killing and they have the highest cytotoxic capacity of all phagocytic cells (Thieblemont et al., 2016, Edwards, 1996). DeChatelet *et al*. reported that phagocytic challenge triggers certain biochemical changes in resting neutrophils, such as increases in oxygen consumption and hexose monophosphate (HMP) shunt activity which generate the NADPH that is subsequently oxidised by the assembled NADPH oxidase for bactericidal activity (DeChatelet, et al., 1976).

Stimulation of neutrophils *in vitro*, whether by phagocytic (e.g. heat-killed bacteria) or by soluble (e.g. fMLP, PMA) agents triggers an increased production of oxidative metabolites, that can be assayed using a variety of techniques which measure the cell's collective oxidative response, such as spectrophotometry, fluorimetry and chemiluminescence (Smith & Weidemann, 1993, Briheim, et al., 1984). Therefore, the experiments described in this Chapter investigated phagocytosis and oxidative burst generation capacities of neutrophils and differentiated PLB-985 cells using flow cytometry and luminol-amplified chemiluminescence assay.

The data presented from the flow cytometric analysis of phagocytosis clearly demonstrated that neutrophils (without cytokine) highly phagocytosed opsonised PI-labeled *S. aureus* (SAPI) and opsonised fluorescent latex beads, and to a lower extent, opsonised non-fluorescent latex beads. Incubation of neutrophils with the cytokine G-CSF or GM-CSF significantly enhanced the levels of phagocytosis of these particles. This agrees with observation by Sémiramoth *et al.,* that priming of neutrophils by proinflammatory cytokines and by LPS optimises their functional responses (Sémiramoth et al., 2009). Furthermore, exposure of blood neutrophils to GM-CSF, like with other priming agents promotes their phagocytic, oxidative and cytotoxic capacities (Edwards et al., 1989). Phagocytosis of fluorescent beads by neutrophils displayed a multi-phasic fluorescence peak signifying different proportions of cells engulfing different numbers of beads of between 1 to 7, with most cells taking up two particles.

Similarly, flow cytometric analysis of differentiated PLB-985 cells also revealed that these cells displayed increasing levels of phagocytosis of SAPI and latex beads in parallel with the increasing levels of their differentiation into neutrophil-like phenotypes. As seen with neutrophils, levels of phagocytosis by differentiated PLB-985 increased also, after incubation with G-CSF or GM-CSF. However, in contrast to

neutrophils, differentiated PLB-985 cells displayed higher phagocytosis of fluorescent beads, followed by SAPI and to a lower extent non-fluorescent beads.

The difference in responses of neutrophils and differentiated PLB-985 cells to SAPI is likely due to difference in the expression of specific receptors between these cell types, as neutrophils express receptors for molecules, such as chemoattractants and opsonins (e.g., fMLP, C5a and IL-8) that facilitate recognition, binding and uptake of the bacteria (Naccache, 2013), which might not be expressed on the surface of differentiated PLB-985 cells. Phagocytosis of inert polystyrene beads is achieved through hydrophobic interactions with the cells, which may partly, explain why the differentiated cells were able to take up these particles. As for blood neutrophils, differentiated PLB-985 cells took up different numbers of fluorescent bead particles ranging from 1 to 6 as evidenced by multiple fluorescent peaks. Most differentiated PLB-985 cells phagocytosed two particles per cell, after day 3 to 6 of culture, under the different culture conditions.

Oxidative burst generation assayed by flow cytometry shows that neutrophils (without cytokine) displayed strong oxidative response when stimulated with PMA and a moderate response was exhibited upon fMLP stimulation. Incubation with cytokine G-CSF or GM-CSF increased the oxidative burst activity significantly in PMA and fMLP stimulated cells, which is particularly higher in the latter. This clearly demonstrated the importance of priming/cytokines to the receptor mediated signalling, such as in fMLP. Latex beads showed a low oxidative burst activity which increased significantly following incubation with both G-CSF and GM-CSF.

Fluorescence distribution patterns of differentiated PLB-985 cells oxidative burst displayed a very similar pattern to that of isolated blood neutrophils. Highest activity was observed in PMA stimulated cells whilst a very weak response was observed in

cells stimulated by fMLP. However, dPLB-985 cells response to both stimulants increased after incubation with cytokine G-CSF or GM-CSF. Latex beads showed lowest oxidative burst activity which also increased slightly when dPLB-985 cells were incubated with G-CSF or GM-CSF. Non-differentiated PLB-985 cells are incapable of oxidative burst generation, as these cells produced negligible amounts of total ROS with both fMLP, PMA or latex beads, which did not change significantly, following incubation with cytokines G-CSF or GM-CSF. Therefore, the small observed ROS could be the result of mitochondrial respiration, and thus strongly suggests that changes associated with differentiation of PLB-985 cells were responsible for their ability to generate the oxidative burst.

Total ROS generation by neutrophils and differentiated PLB-985 cells measured using luminol-enhanced chemiluminescence assay revealed striking consistency with the data obtained from flow cytometric measurements, as well as similarity between neutrophils and differentiated PLB-985 cells. Neutrophils (without cytokine) produced high total chemiluminescence in response to PMA stimulation, which was low in response to fMLP. Following incubation with GM-CSF however, the fMLP induced chemiluminescence was highly and significantly increased, whilst only slight and statistically-insignificant increase was observed in PMA-induced cells. This is not surprising, because PMA being membrane permeable, does not require priming to activate neutrophils, though their response to PMA can be accelerated by priming (DeChatelet et al., 1976). The unstimulated neutrophils expressed very little and insignificant total chemiluminescence.

PMA, a structural analog of diacylglycerol (DAG) activates neutrophil response exogenously by direct activation of protein kinase C (PKC), hence activation of NADPH oxidase by PMA is independent of receptor binding or generation of second messages (Edwards, et al., 1990). Whereas the bacterial peptide fMLP activates

neutrophils via the formyl peptide receptors (FPR) that may be present on the granules, and priming is required to mobilise these granules to the cell surface (Hallett & Lloyds, 1995). Normally, *in vivo* neutrophil activation occurs by receptor-coupled mechanisms, which usually, are regulated by pre-exposure to priming agents such as GM-CSF, that enhance their functional responsiveness (Edwards et al., 1990).

Reports of chemiluminescence studies in patients with MPO deficiencies have revealed that luminol-amplified light emission by neutrophils is entirely dependent on the MPO-H_2O_2 system (Dahlgren & Stendahl, 1983). The chemotactic peptide fMLP induces a biphasic chemiluminescence peak in normal neutrophils with the initial sharp peak of activity within 2-3 min and the second peak at around 5-7 min after addition of stimulus. In contrast, PMA induces a single response peak, reaching maximum at around 5-7 min also, as shown in Figure 5.17. The first peak of the fMLP response is a result of extracellular oxidants generation, while the second peak is a result of intracellular oxidants caused by internalisation of fMLP by endocytosis or entrance of luminol into the cells to become oxidised intracellularly (Briheim, et al., 1984). Most of the PMA induced response is believed to be due to intracellular oxidants (Dahlgren, 1987). While differentiated PLB-985 did not produce any chemiluminescence response after fMLP stimulation, PMA showed a single response peak similar to that of blood neutrophils. Interestingly, non-differentiated PLB-985 cells did not produce chemiluminescence in response to both stimulants, another strong indication that differentiation of PLB-985 cells was in deed responsible for the PMA observed chemiluminescence responses in Figure 5.19.

In conclusion, these data show that isolated blood neutrophils have variable phagocytic and oxidative burst generation capacities to a variety of particles and stimuli, and the neutrophil-like differentiated PLB-985 cells expressed similar but weaker phagocytic and oxidative burst activities in response to the same particles

and stimuli. This suggests that differentiated PLB-985 cells, like the isolated mature blood neutrophils, can display functions of professional phagocytosis and oxidative killing.

Chapter 6: Effect of differentiation of PLB-985 cells on expression of cell surface markers, chemotaxis and intracellular signalling

6.1 Introduction

Neutrophil functions are mediated by several cell surface receptors which upon activation, trigger complex intracellular signal transduction pathways. The microbicidal activity of neutrophils is dependent upon effective pathogen recognition and binding, mediated by many classes of receptors expressed on their plasma membranes, including adhesion receptors (e.g. selectins and integrins), immunoglobulin receptors (FcγRs), G-protein-coupled receptors (GPCRs), cytokine receptors and Toll-like receptors (Bruhns, 2012; Migeotte et al., 2006). Activation of these receptors triggers cellular responses required for pathogen elimination, such as chemotactic transmigration, phagocytosis, degranulation and oxidative burst generation (Futosi et al., 2013).

Circulating blood neutrophils constitutively express the β_2-integrin adhesion receptors, and their expression increases rapidly following activation (Drayson et al., 2001; Repo et al., 1993). Integrins are heterodimeric transmembrane glycoproteins expressed on almost all mammalian cells. The most important integrins expressed on neutrophils belong to the β_2-integrin family, including the CD11b/CD18 heterodimer or CR3 or Mac-1 complex (Futosi et al., 2013). CD11b/CD18 mediates cell-cell interactions and firm adhesion of neutrophils to the endothelium during transcellular migration through vessel walls and intestinal tissues (Siddiqi et al., 2001). Elevated expression of CD11b leads to an increased recruitment of neutrophils to the infection

sites (van Eeden et al., 1999). CD11b is described as an early expressed marker during granulocyte differentiation and maturation (Drayson et al., 2001).

The LPS receptor, CD14 is a 55 KDa plasma membrane glycoprotein expressed strongly on monocytes and macrophages, but in lower amounts on granulocytes (Simmons et al., 1989). However, its expression on resting neutrophils can be rapidly upregulated upon stimulation with cytokines, such as TNF, G-CSF and GM-CSF (Fleck, 2005). CD14 is a multifunctional receptor that can act as an opsonic receptor by binding bacterial lipopolysaccharide (endotoxin) to promote phagocytosis of bacteria and LPS-coated particles, and trigger inflammatory responses by macrophages. In addition, it is an important receptor in the clearance of apoptotic cells, where it recognises and interacts with apoptotic cells, leading to their phagocytic uptake. However, contrary to LPS, uptake of apoptotic cells does not provoke the macrophages to release pro-inflammatory cytokines (Devitt et al., 1998; Wright et al., 1991).

The immunoglobulin receptors are also plasma membrane glycoproteins called the Fcγ receptors because they recognise and bind the Fc domain of IgG. They primarily recognise IgG-opsonised pathogens and particles, but are also involved in immune complex-coupled inflammatory responses (Futosi et al., 2013). Three classes of FcγRs have been identified: FcγRIII (CD16), FcγRII (CD32) and FcγRI (CD64) (Hazan-Eitan et al., 2006; Edwards, 1994). Human neutrophils constitutively express low affinity Fcγ receptors; FcγRIII and FcγRII, but the high affinity FcγRI is not expressed on resting neutrophils, unless activated by cytokines, such as GM-CSF and interferon-γ (Perussia et al., 1983). The low affinity FcγRs bind monomeric IgG with low affinity and are the most abundant FcγRs on neutrophils. FcγRIII (CD16) receptors are also present intracellularly in numerous small vesicles (120-180nm) distributed within the cytoplasm. These vesicles represent an internal pool of Fc

receptors that can readily be mobilised and transported to the plasma membrane upon activation (Jost et al., 1990).

Extravasation and chemotaxis of neutrophils are critical events and prerequisites for their efficient microbicidal and clearance roles, because they allow neutrophils to be rapidly recruited to the sites of infection or inflammation. The chemotactic migration of neutrophils *in vivo,* occurs towards a gradient of chemoattractants (see Figure 1.3), such as inflammatory mediators (e.g. fMLP and IL-8) or other chemotactic factors (e.g. C5a, casein and activated serum) (Singh et al., 2014). These attractants are released during inflammation, and in turn, trigger rapid morphological changes in neutrophils from spherical to polarized shape, exhibiting a front, leading lamella and a contracted, trailing tail. This is followed by re-orientation of actin filaments (for detection of the chemotactic gradient) and subsequently, directed migration up the gradient (Nuzzi et al., 2015; Hauert et al., 2002).

The complex networks of signalling systems in neutrophils are mediated by plasma membrane receptors, downstream G-protein and/or tyrosine kinases, including lipid-activated kinases (e.g. phospholipase C and phosphatidylinositol 3-kinase), and their activation regulates activation of various neutrophil functions. Apart from rapid activation of specific enzymes via post-translational modifications, these signalling networks activate transcription factors, hence trigger transcript to modify functional responses (Futosi et al., 2013; Naccache, 2013).

Results in the previous Chapter showed that neutrophils and differentiated PLB-985 cells expressed certain receptor-mediated processes, such as phagocytosis and oxidative burst generation. This Chapter will therefore, investigate the expression of some cell surface markers on isolated blood neutrophils and on neutrophil-like differentiated PLB-985 cells to identify their functional responses, and compare the

expression levels between the two cell types, to get further insights into the functional and structural similarities of differentiated PLB-985 cells to mature blood neutrophils.

The aims of the work described in this Chapter therefore are:

1. To compare the expression of the adhesion receptor CD11b, the LPS receptor CD14 and the immunoglobulin receptor CD16 on isolated blood neutrophils and differentiated PLB-985 cells.
2. To measure the transmigration and chemotaxis of neutrophils and differentiated PLB-985 cells in response to various chemoattractants.
3. To determine the activation status of the STAT3 intracellular signalling pathway in differentiated PLB-985 cells.

6.2 Methods

Detailed methodologies are described in Chapter 2. Expression of plasma membrane surface markers on neutrophils and differentiated PLB-985 cells was analysed by flow cytometry. Cells were prepared, fixed with 4% PFA and stained with specific conjugated antibodies to CD11b, CD14 and CD16, including isotype-matched conjugated IgG as control. For intracellular CD16 expression, cells were permeabilised using 0.1% saponin (in PBS) prior to staining, as described in the methods Chapter. Expression of the various surface receptors was then analysed using the flow cytometer.

Chemotaxis of neutrophils and differentiated PLB-985 cells was analysed by a transwell assay, using Millipore hanging inserts, fitted with 3 µm pore sized filters. Cells were prepared and loaded onto the hanging inserts, suspended in a 24-well

poly-hema coated tissue culture plate, containing the chemoattractants; fMLP (10^{-8}M), IL-8 (0.1µg/mL), casein (0.5 mg/mL), or zymosan A activated serum (1µg/mL) as described in Methods. Cells that transmigrated into each well were counted using a Multisizer 3 cell coulter counter, following a 1:1000 dilution with Isoton II and the results were expressed as percentages of the total number of cells originally added.

The activation status of the STAT3 intracellular signalling pathway in differentiated PLB-985 cells was determined by western blot analysis. Protein extracts were prepared as described in the Methods and probed for the expression of activated STAT3 using relevant anti-human antibodies.

6.3 Results

6.3.1 Expression of surface CD11b on neutrophils

Freshly-isolated neutrophils from healthy donors were incubated for 30 min in the absence or presence of G-CSF (10ng/mL) or GM-CSF (5ng/mL) and then stained with saturating PE-conjugated CD11b antibody or isotype-IgG control. Cells were then analysed for the expression of CD11b using the flow cytometer. Figure 6.1 showed that CD11b surface receptor was highly expressed on neutrophils with no cytokine (NC) (MFI 1252.03 ± 54.95) and this significantly increased ($p \leq 0.05$) after incubation with the cytokines G-CSF (MFI 1470.55 ± 61.74) or GM-CSF (MFI 1475.73 ± 82.46). The percent positive cells in the absence of cytokine (NC) was also high (80.01% ± 2.01%), which increased significantly ($p \leq 0.05$) following incubation with G-CSF (88.82% ± 1.22%) or GM-CSF (90.29% ± 0.94%). Representative traces of CD11b expression on neutrophils under these conditions are shown in Figure 6.4.

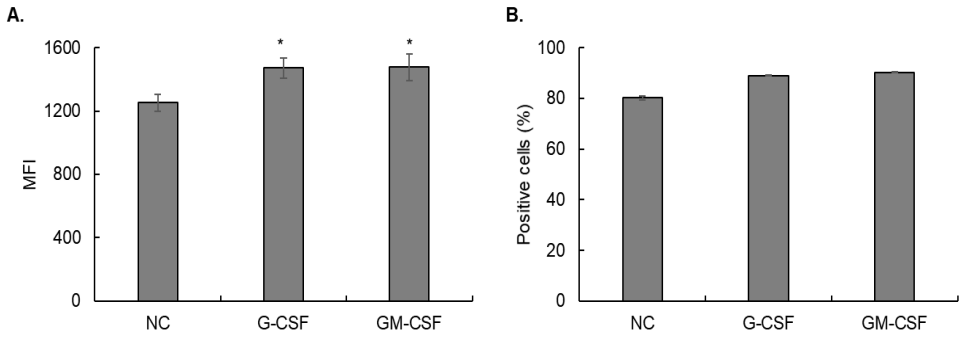


Figure 6.1 Expression of CD11b on the surface of neutrophils. Freshly-isolated neutrophils incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were prepared and stained with saturating PE-conjugated CD11b antibody or isotype-IgG control. The cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of surface CD11b antigen using the flow cytometer. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

6.3.2 Expression of surface CD14 on neutrophils

Freshly-isolated neutrophils from healthy donors were incubated in the absence or presence of G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min and stained with saturating PerCP-conjugated CD14 antibody or isotype-IgG control. Cells were then analysed for the expression of CD14 surface antigen using the flow cytometer. CD14 expression was very low on neutrophils in the absence of cytokines (NC) (MFI 35.75 ± 2.38) as shown on Figure 6.2. This was, however, significantly increased ($p \leq 0.05$) upon incubation with cytokine G-CSF (MFI 44.54 ± 2.21) or GM-CSF (MFI 45.04 ± 1.93). Similarly, small percentage of the cells stained positive for CD14 (15.89% ± 2.81%), and this proportion increased in cells incubated with G-CSF (19.14.27% ± 3.84%, NS) and GM-CSF (26.63% ± 2.49%, $p \leq 0.05$). This confirms previous reports that CD14 is expressed at low levels on granulocytes, but treatment with pro-inflammatory cytokines, such as TNF, G-CSF and GM-CSF can enhance its expression (Fleck, 2005). Representative traces of CD14 expression on neutrophils under these conditions are shown in Figure 6.4.

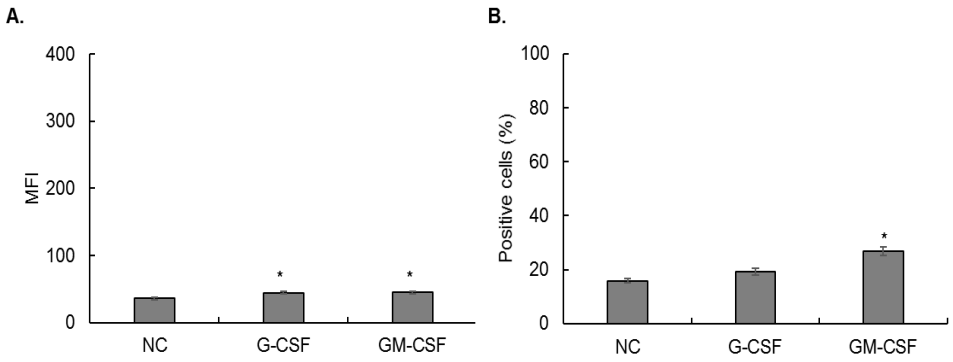


Figure 6.2 Expression of CD14 on the surface of neutrophils. Freshly-isolated neutrophils incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were prepared and stained with saturating PerCP-conjugated CD14 antibody, including isotype-IgG control. Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of CD14 surface marker using the flow cytometer. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05 (paired, two-tailed student's t-test).

6.3.3 Expression of surface CD16 on neutrophils

Freshly-isolated neutrophils from healthy donors incubated in the absence and presence of G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were stained with saturating FITC-conjugated CD16 antibody or isotype-matched IgG control. Cells were then analysed for expression of CD16 surface marker using the flow cytometer.

As shown in Figure 6.3, neutrophils incubated in the absence of cytokine (NC) expressed high levels of surface CD16 (MFI 145.71 ± 18.72), which increased following incubation with the cytokines G-CSF (MFI 166.42 ± 17.11) or GM-CSF (MFI 182.13 ± 12.26), but these increases did not reach statistical significance ($p \leq 0.05$). There was however, a high percent positive cells for surface CD16 (75.74% ± 3.96%), which increased significantly ($p \leq 0.05$) in cells incubated with cytokine G-CSF (82.36% ± 2.09%) or GM-CSF (89.88% ± 2.74%). CD16 is constitutively expressed at high concentrations on neutrophils. Representative traces of surface CD16 expression on neutrophils under these conditions is shown in Figure 6.4.

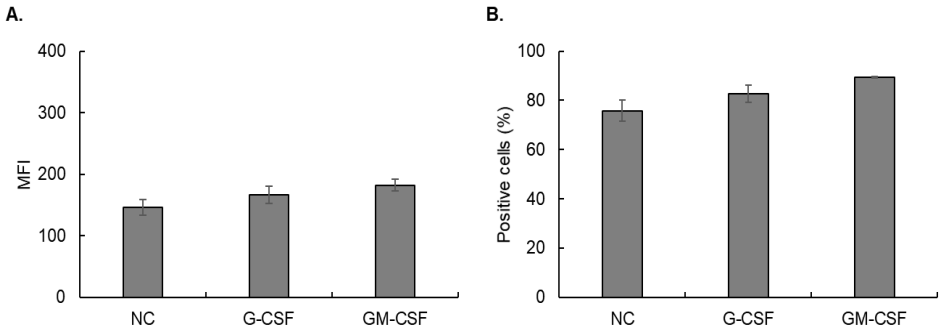


Figure 6.3 Expression of CD16 on the surface of neutrophils. Freshly-isolated neutrophils incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were prepared and stained with saturating FITC-conjugated CD16 antibody or isotype-IgG control. Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of CD16 surface antigen using the flow cytometer. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. Data are expressed as means of MFI or percent total cells (± SEM, n=3).

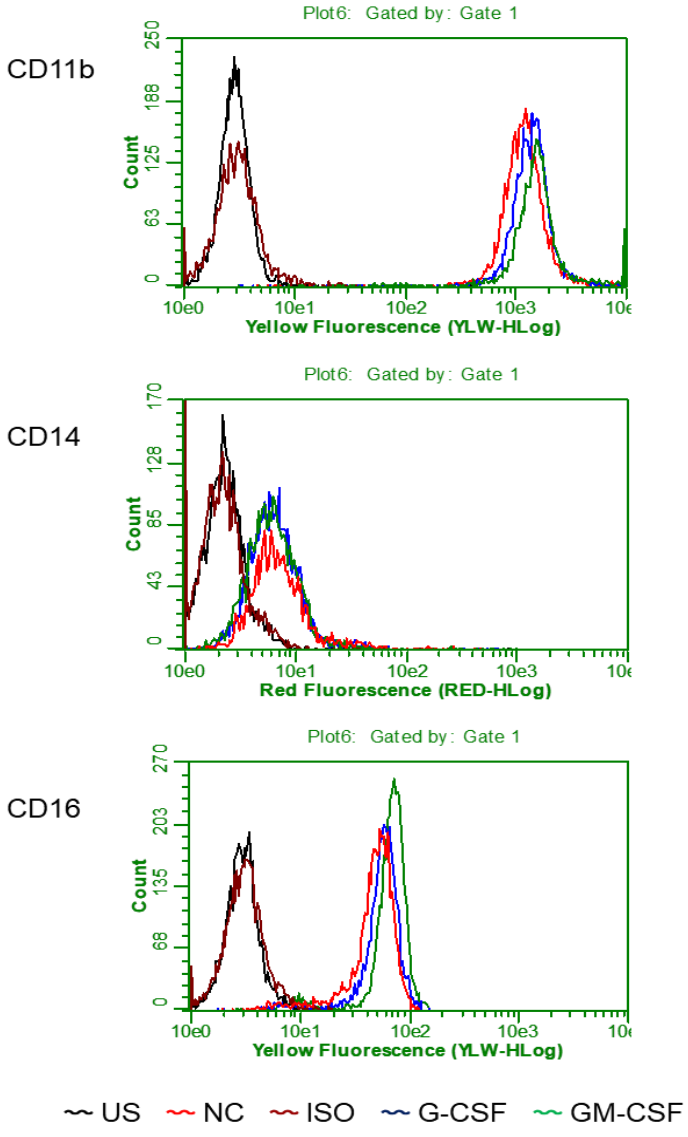


Figure 6.4 Representative flow cytometer traces for CD11b, CD14 and CD16 expression on the surface of neutrophils. Freshly-isolated neutrophils incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were prepared and stained with saturating conjugated antibodies or isotype-IgG control (ISO), including unstained (US) samples to gate for negative fluorescence. Cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of the surface antigens using the flow cytometer.

6.3.4 Expression of surface CD11b on differentiated PLB-985 cells

PLB-985 and dPLB-985 cells cultured for 6 days in unchanged media were stained with saturating PE-conjugated CD11b antibody or isotype-matched IgG control and then analysed for expression of CD11b marker using the flow cytometer. Figure 6.5A-B shows the expression of CD11b on PLB-985 and dPLB-985 cells. Very low and insignificant expression of the surface receptor was measured on the non-differentiated PLB-985 cells (MFI 13.01 ± 2.69 – 16.00 ± 2.81) over the 6 days culture period.

However, following differentiation into neutrophil-like cells, expression of this receptor significantly ($p \leq 0.05$) increased in these cells; day 3 (MFI 37.87 ± 5.56), day 5 (MFI 45.22 ± 1.19) and day 6 (MFI 37.99 ± 5.33), with day 5 showing the highest expression. The percent positive cells showed a similar and parallel trend for cells grown under both culture conditions. This shows that differentiation induces the expression of surface CD11b on differentiated PLB-985 cells, but this was lower than the expression levels observed on neutrophils (Figure 6.1). Representative traces of surface CD11b expression on PLB-985 and dPLB-985 cells is shown in Figure 6.5C.

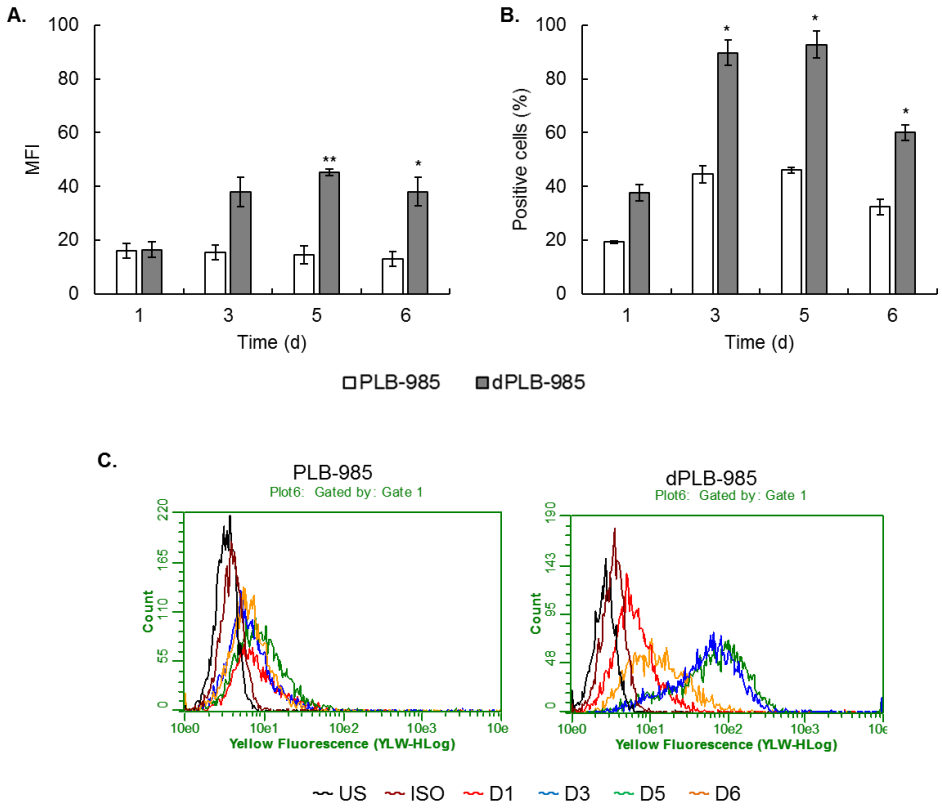


Figure 6.5 Effect of differentiation of PLB-985 cells on the expression of CD11b. PLB-985 and dPLB-985 cells cultured for 6 days in unchanged media were prepared and stained with saturating PE-conjugated CD11b antibody or isotype-IgG control. Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for the expression of surface CD11b using flow cytometry. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. (C) Representative flow cytometer traces of CD11b expression. Data were expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05 (paired, one-tailed student's t-test).

6.3.5 Expression of surface CD14 on differentiated PLB-985 cells

PLB-985 and dPLB-985 cells cultured for 6 days in unchanged media were stained with saturating PerCP-conjugated CD14 antibody or isotype-matched IgG control. Cells were then analysed for expression of CD14 receptor using the flow cytometer. As shown in Figure 6.6A-B, expression of CD14 surface marker on the non-differentiated PLB-985 cells is very low and statistically insignificant (MFI 10.37 ± 2.16 – 14.27 ± 0.35) over the culture period. This increased slightly and insignificantly following differentiation into neutrophil like cells (D5: MFI 16.83 ± 1.03; D6: MFI 15.15 ± 2.32). The same trend was also observed for the percent positive cells, but this increased significantly ($p \leq 0.05$) on day 3 (48.22% ± 2.09%) and day 5 (53.71% ±3.64%). Therefore, differentiation increased the expression of surface CD14 on PLB-985 cells, but as with neutrophils, this was very low. Figure 6.6C shows representative traces of surface CD14 expression on PLB-985 and dPLB-985 cells.

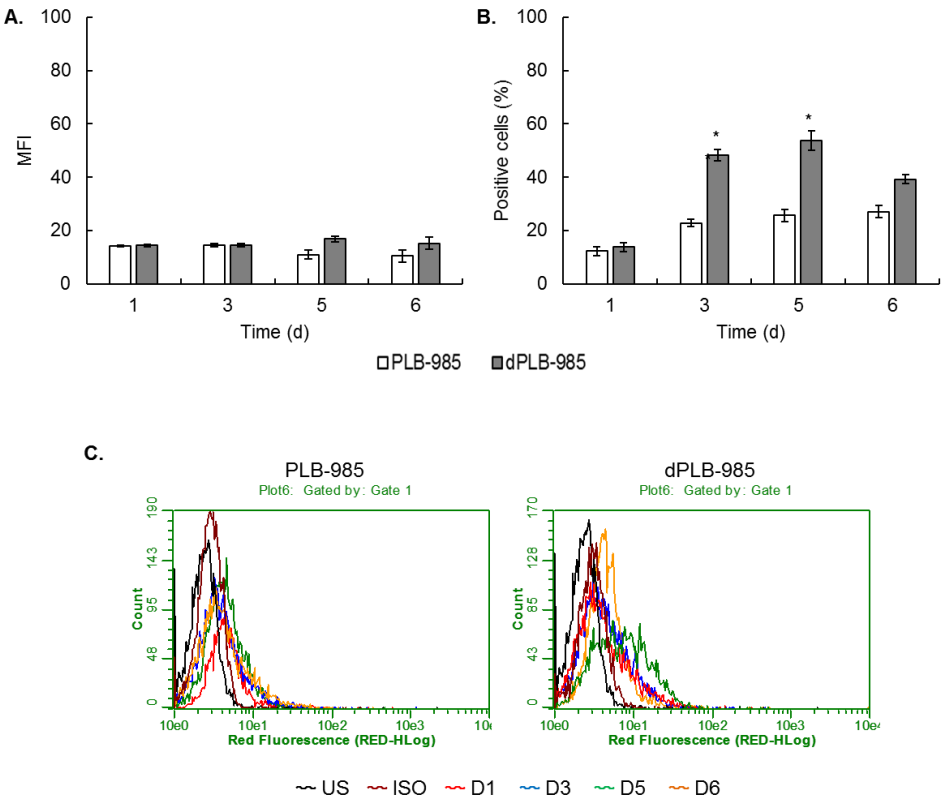


Figure 6.6 Effect of differentiation of PLB-985 cells on the expression of CD14. PLB-985 and dPLB-985 cells cultured for 6 days in unchanged media were prepared and stained with saturating PerCP-conjugated CD14 antibody or isotype-IgG control. Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of surface CD14 using flow cytometry. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. (C) Representative flow cytometer traces of CD14 expression. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05 (paired, one-tailed student's t-test).

6.3.6 Expression of surface CD16 on differentiated PLB-985 cells

PLB-985 and dPLB-985 cells cultured for 6 days in unchanged media were stained with saturating FITC-conjugated CD16 antibody or isotype-matched IgG control. Cells were then analysed for expression of surface CD16 receptor by flow cytometry, shown in Figure 6.7A-B. There was very low or no expression of CD16 on non-differentiated PLB-985 cells (MFI 9.85 ± 1.68 – 13.07 ± 1.14) throughout the culture period. However, differentiated PLB-985 cells showed a low but significant ($p \leq 0.05$) increase in the expression of this receptor on day 3 (MFI 14.00 ± 1.51), day 5 (MFI 17.27 ± 2.04) and day 6 (MFI 15.93 ± 1.33) of culture. There was also a significant increase in the percent positive cells for CD16 on day 3 (9.98% ± 1.60%) and day 5 (13.90% ±1.17%). Therefore, differentiation of PLB-985 cells induced a small expression of CD16, but this was very low compared to blood neutrophils. Representative traces of CD16 expression on PLB-985 and dPLB-985 cells are shown in Figure 6.7C.

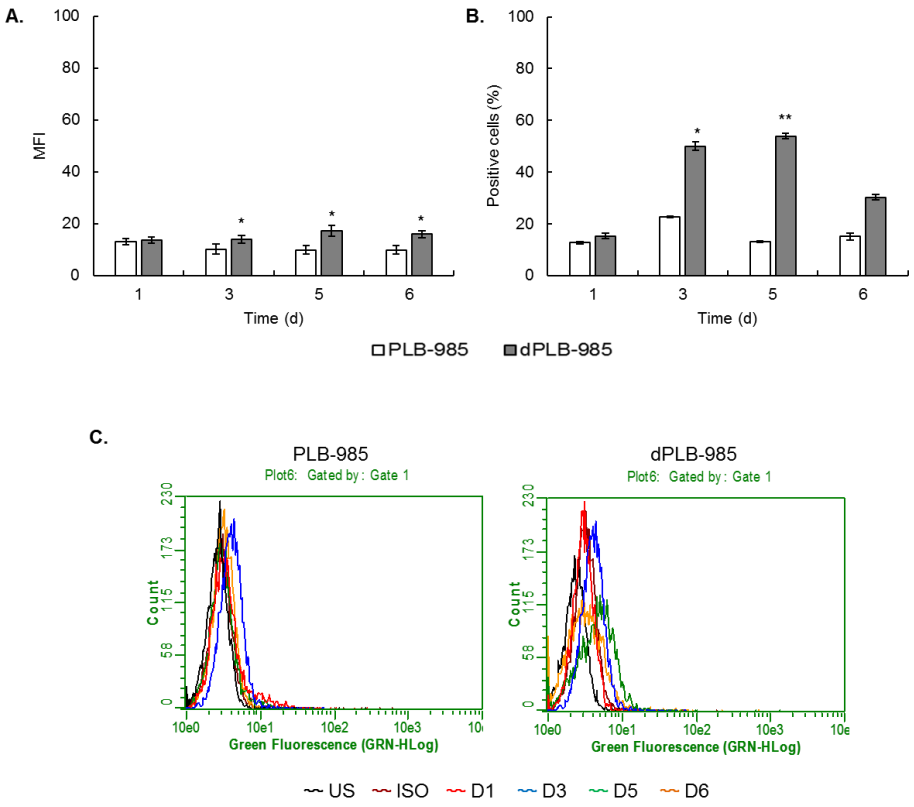


Figure 6.7 Effect of differentiation of PLB-985 cells on the expression of CD16. PLB-985 and dPLB-985 cells cultured for 6 days in unchanged media were prepared and stained with saturating FITC-conjugated CD16 antibody or isotype-IgG control. Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of surface CD16 using flow cytometry. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. (C) Representative flow cytometer traces of CD16 expression. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, one-tailed student's t-test).

6.3.7 Effects of media changes and cytokine addition on the expression of CD11b on dPLB-985 cells

dPLB-985 cells differentiated under the optimised conditions involving media changes and cytokine supplementation for 6 days as described in Figure 3.16, were incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min and stained with saturating FITC-conjugated CD11b antibody or isotype-IgG control. Cells were then analysed for expression of surface CD11b receptors by flow cytometry.

Figure 6.8 shows CD11b expression on the surface of differentiated PLB-985 cells. The expression level of the receptor in the absence of the cytokine (NC) was low (MFI 17.18 ± 1.23 – 23.97 ± 2.55) during this culture period. This was, even lower than the level of expression measured on dPLB-985 cultured in unchanged media (see Figure 6.5), this was likely due to the difference in the antibody used: in these experiments, the antibody is FITC-conjugated while in Figure 6.5, PE-conjugated CD11b was used. Following incubation with the cytokines, this expression increased from day 3: G-CSF (D3: MFI 25.32 ± 1.62; D5: MFI 33.71 ± 2.84; D6: MFI 32.58 ± 2.68) and GM-CSF (D3: MFI 24.03 ± 3.20; D5: MFI 34.73 ± 2.49; D6: MFI 32.95 ± 3.06), but only on days 5 and 6 did these increases reached statistical significance ($p \leq 0.05$). Representative traces of CD11b surface marker expression on differentiated PLB-985 cells under these conditions are shown in Figure 6.9.

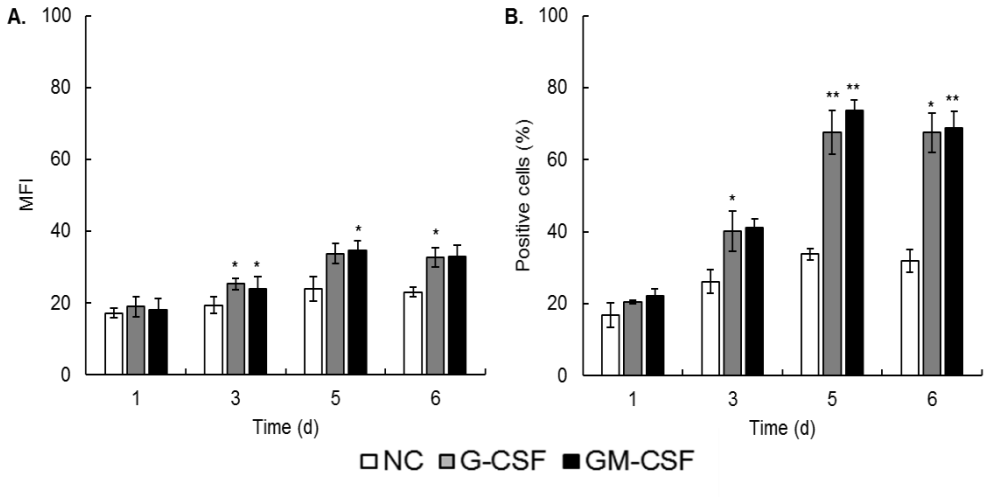


Figure 6.8 Effect of media changes and cytokine addition on the expression of surface CD11b on dPLB-985 cells. Cells differentiated under the optimised conditions for 6 days (see Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were prepared and stained with saturating FITC-conjugated CD11b antibody or isotype-IgG control. Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of CD11b using flow cytometry. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

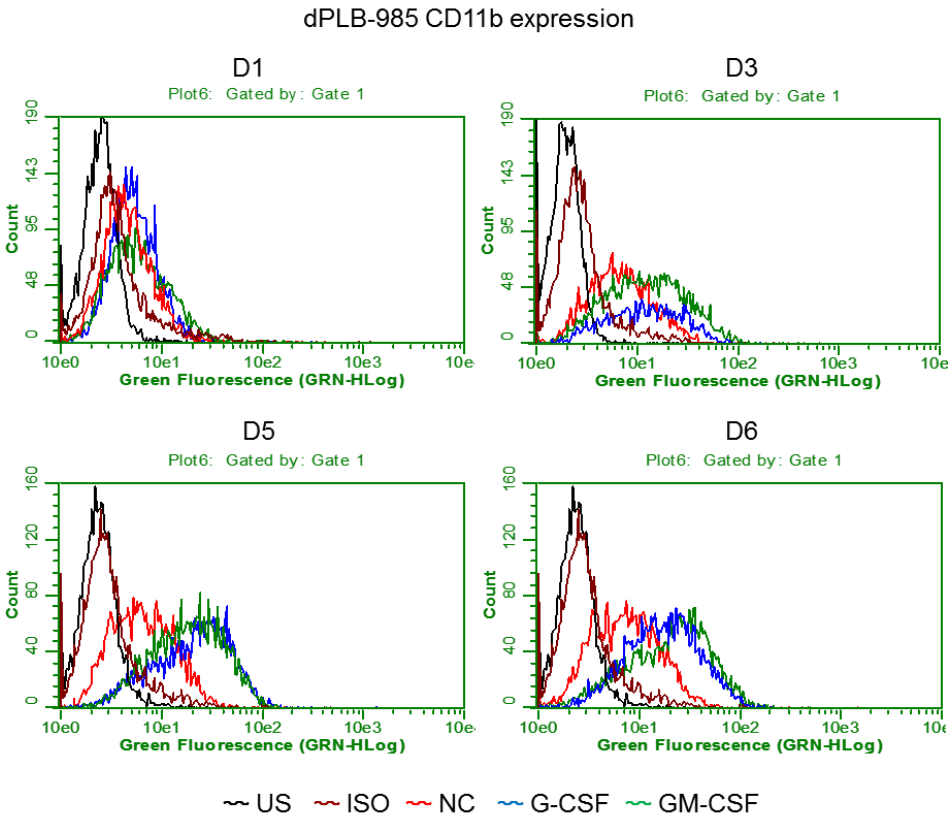


Figure 6.9 Representative flow cytometer traces of CD11b expression on dPLB-985 cells. Cells differentiated under the optimised conditions for 6 days (described in Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, were prepared and stained with saturating FITC-conjugated CD11b antibody or isotype-IgG control (ISO) including unstained (US) samples (to gate for negative fluorescence). Cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of CD11b using flow cytometry.

6.3.8 Effects of media change and cytokine addition on the expression of CD14

dPLB-985 cells differentiated under the optimised conditions involving media changes and cytokine supplementation for 6 days as described in Figure 3.16, were incubated in the absence or presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL), and stained with saturating PerCP-conjugated CD14 antibody or isotype-IgG control. Cells were then analysed for expression levels of CD14 receptors by flow cytometry.

As shown in Figure 6.10, expression of CD14 on the surface of differentiated PLB-985 cells did not significantly increase over 6 days culture with media changes alone (NC) (MFI 14.04 ± 0.83 – 15.28 ± 1.78) when compared to dPLB-985 cells grown in unchanged media (Figure 6.6). This however, increased from day 3 following incubation with the cytokines G-CSF (D3: MFI 21.64 ± 1.95; D5: MFI 22.67 ± 1.79; D6: MFI 19.52 ± 2.87) or GM-CSF (D3: MFI 22.09 ± 1.52; D5: MFI 25.48 ± 1.40; D6: MFI 22.62 ± 1.28), but only GM-CSF levels reached statistical significance ($p \leq 0.05$) on days 5 and 6. Similar to isolated blood neutrophils, expression of surface CD14 on differentiated PLB-985 cells was low. Figure 6.11 shows representative traces of CD14 surface marker expression on differentiated PLB-985 cells under these experimental conditions.

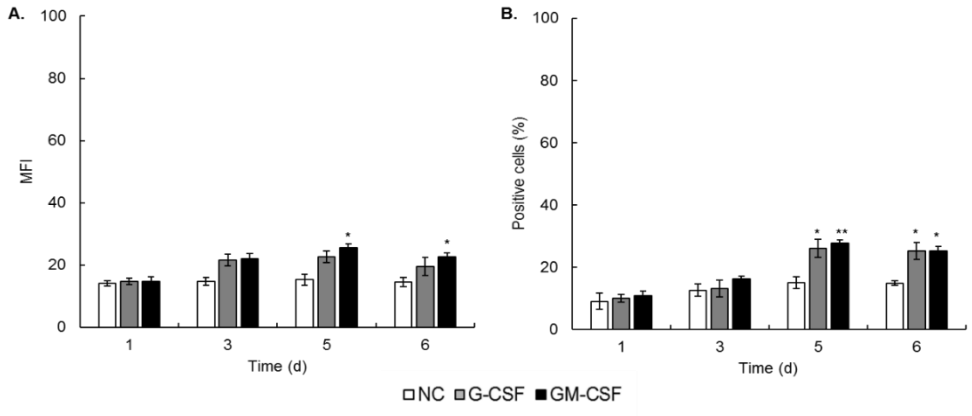


Figure 6.10 Effect of media changes and cytokine addition on the expression of CD14 on dPLB-985 cells. Cells differentiated under the optimised conditions for 6 days (described in Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, were prepared and stained with saturating PerCP-conjugated CD14 antibody or isotype-IgG control. Cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of CD14 using flow cytometry. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

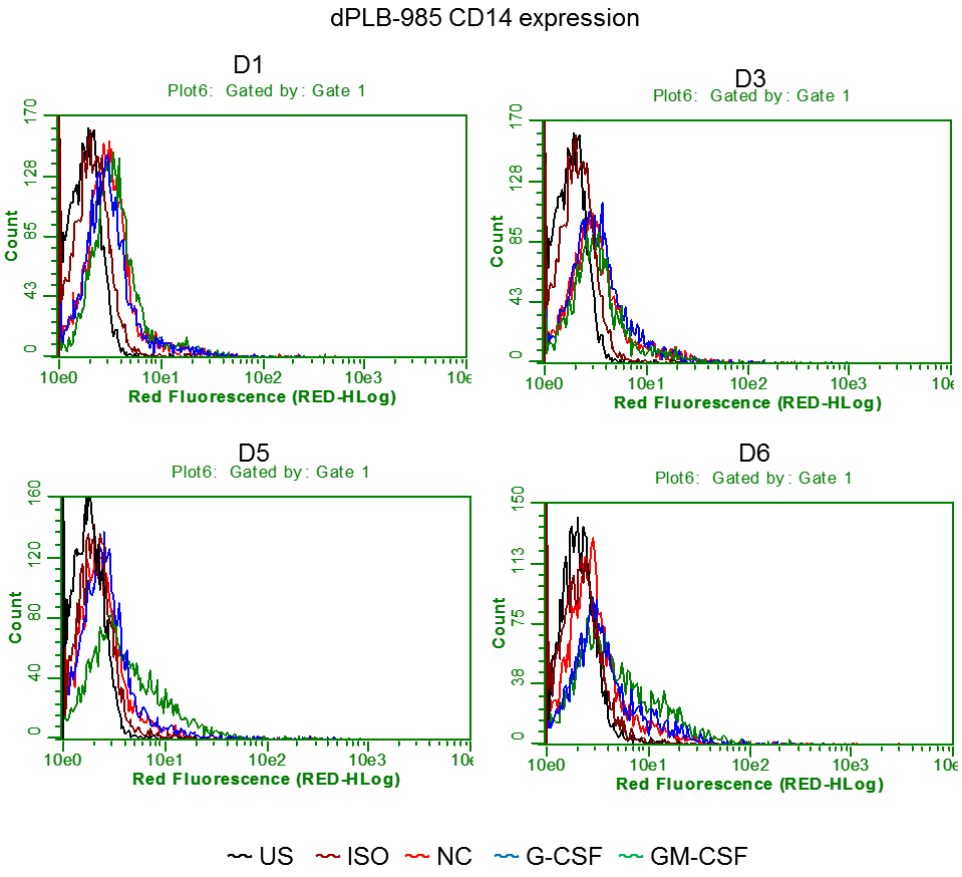


Figure 6.11 Representative flow cytometer traces of surface CD14 expression on dPLB-985 cells. Cells differentiated under the optimised conditions for 6 days (see Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were prepared and stained with saturating PerCP-conjugated CD14 antibody or isotype-IgG control (ISO) including unstained (US) samples (to gate for negative fluorescence). Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of CD11b using flow cytometry.

6.3.9 Effects of media changes and cytokine addition on the expression of CD16

dPLB-985 cells differentiated under the optimised conditions involving media changes and cytokine supplementation for 6 days as described in Figure 3.16, were incubated in the absence or presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL), and stained with saturating FITC-conjugated CD16 antibody or isotype-IgG control. Cells were then analysed for expression levels of CD16 receptor by flow cytometry.

The expression of CD16 on the surface of differentiated PLB-985 cells is shown in Figure 6.12. There was a slight increase in expression of this receptor on dPLB-985 cells in cultures with media changes alone (NC) (MFI 13.53 ± 0.24 - 14.19 ± 0.63) over the culture period, when compared to culture with unchanged media (see Figure 6.7). This expression was also increased from day 5 following incubation with cytokine G-CSF (D5: MFI 17.79 ± 2.17; D6: MFI 16.22 ± 0.75) or GM-CSF (D5: MFI 18.55 ± 3.05; D6: MFI 17.57 ± 2.40). These increases in MFI however, did not reach statistical significance ($p \leq 0.05$), but there were significantly more positive cells after days 5 and 6 of culture in the presence of the cytokines. Overall, expression of CD16 on the surface of differentiated PLB-985 remained very low under all experimental conditions in comparison to isolated blood neutrophils. Representative traces of CD16 surface marker expression on dPLB-985 cells under these conditions is shown on Figure 6.13.

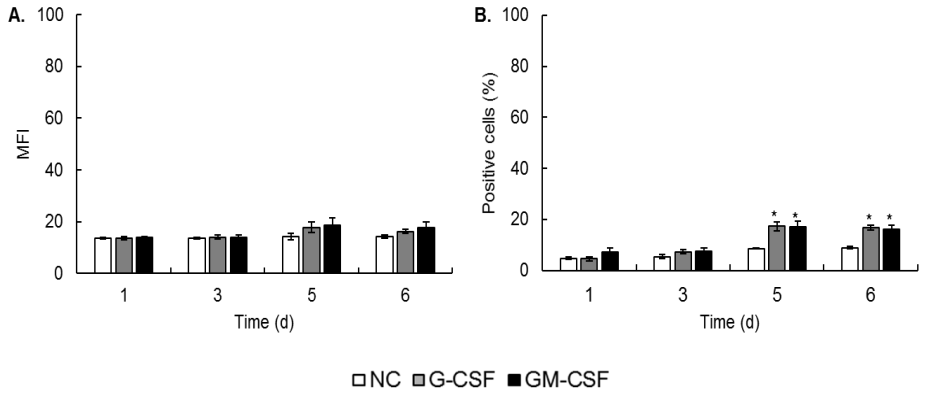


Figure 6.12 Effects of media changes and cytokine addition on the expression of CD16 on dPLB-985 cells. Cells differentiated under the optimised conditions for 6 days (see Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, were prepared and stained with saturating FITC-conjugated CD16 antibody or isotype-IgG control. Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for the expression of CD16 using flow cytometry. (A) Mean fluorescence intensity (MFI) (B) Percent positive cells. Data are expressed as means of MFI or percent total cells (± SEM, n=3), * = p≤0.05 (paired, two-tailed student's t-test).

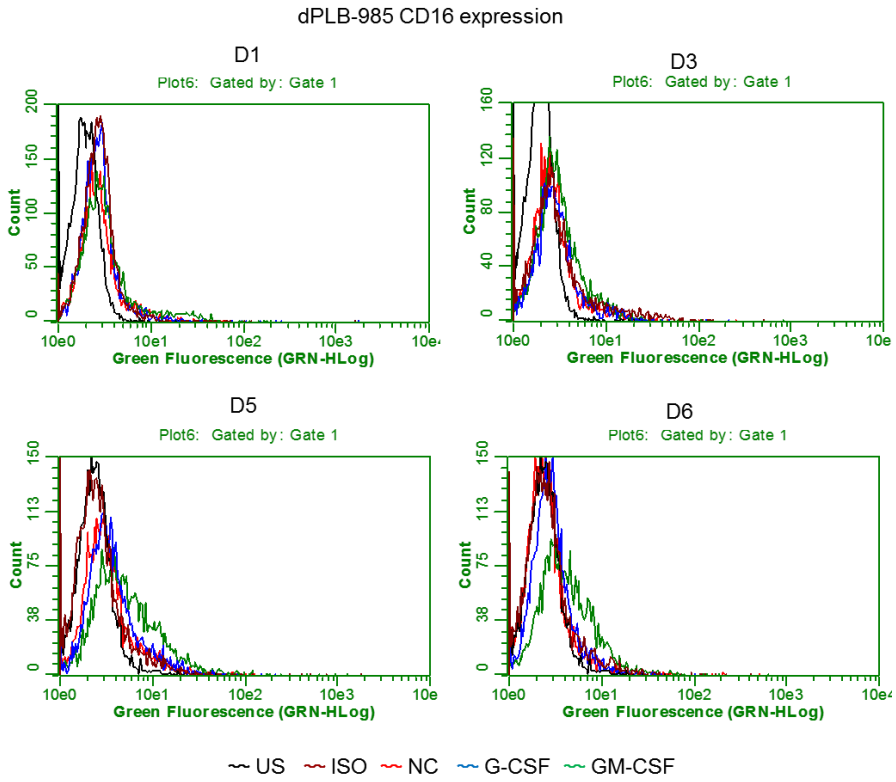


Figure 6.13 Representative flow cytometer traces of CD16 expression on dPLB-985 cells. Cells differentiated under the optimised conditions for 6 days (described in Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, were prepared and stained with saturating FITC-conjugated CD16 antibody or isotype-IgG control (ISO), including unstained (US) sample (to gate for negative fluorescence). Stained cells were washed with PBS (+BSA) and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%), pelleted and re-suspended in PBS (+BSA). Cells were then analysed for expression of CD11b using flow cytometry.

6.3.10 Expression of intracellular CD16 in dPLB-985 cells

The above experiments showed that surface levels of CD16 receptors were low on differentiated PLB-985 cells, even in those cultures with cytokine addition and which showed good morphological features of neutrophils (Chapters 3 and 4). CD16 receptors can be located on the cell surface and also intra-cellularly within the granules of human neutrophils (Jost et al., 1990). Therefore, it was important to determine if there were any intracellular pools of CD16 in differentiated PLB-985 cells.

dPLB-985 cells differentiated under the optimised conditions, involving media changes and cytokine supplementation for 6 days as described in Figure 3.16 were therefore, incubated in the absence and presence of G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min. The cells were prepared, fixed, permeabilized with 0.1% saponin (in PBS) and stained with saturating FITC-conjugated CD16 antibody or isotype-IgG control as described in the Methods. The permeabilized cells were then analysed for intracellular CD16 receptor expression by flow cytometry. Figure 6.14 shows that there was very low, if any, expression of intracellular CD16 in differentiated PLB-985 cells without cytokine (NC, MFI 11.91 ± 0.58 - 14.86 ± 0.72), as well as with cytokine G-CSF (MFI 12.55 ± 0.50 - 15.55 ± 0.59) or GM-CSF (MFI 13.30 ± 0.40 - 15.42 ± 0.69) over the 6 days culture period. The very small increase in expression observed following G-CSF and GM-CSF supplementations, also did not reach statistical significance ($p \leq 0.05$).

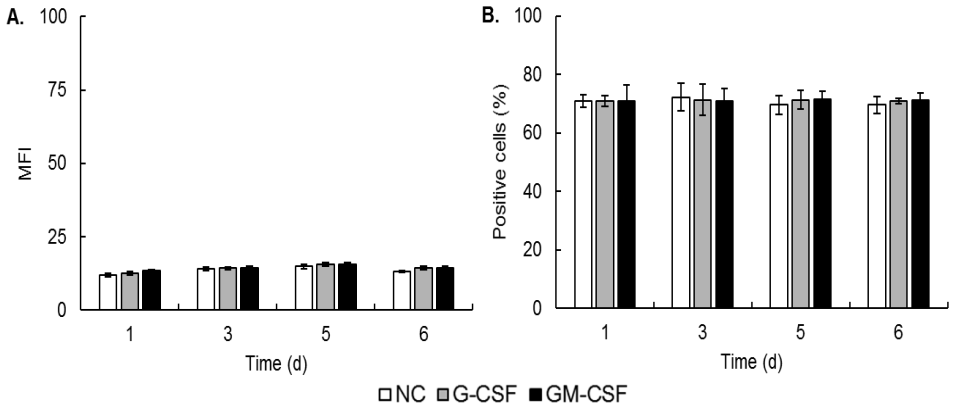


Figure 6.14 Expression of intracellular CD16 in differentiated PLB-985 cells. Cells differentiated under the optimised conditions for 6 days (described in Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were prepared and fixed by addition of equal volumes of PBS (+BSA) and PFA (4%). The cells were permeabilised with PBS (+0.1% saponin) and stained with saturating FITC-conjugated CD16 antibody or isotype-IgG control. Cells were then washed with PBS (+BSA), pelleted, re-suspended in PBS (+0.1% saponin) and analysed for the expression of intracellular CD16 using flow cytometry. (A) Mean fluorescence intensity (MFI). (B) Percent positive cells. Data are expressed as means of MFI or percent total cells (± SEM, n=3).

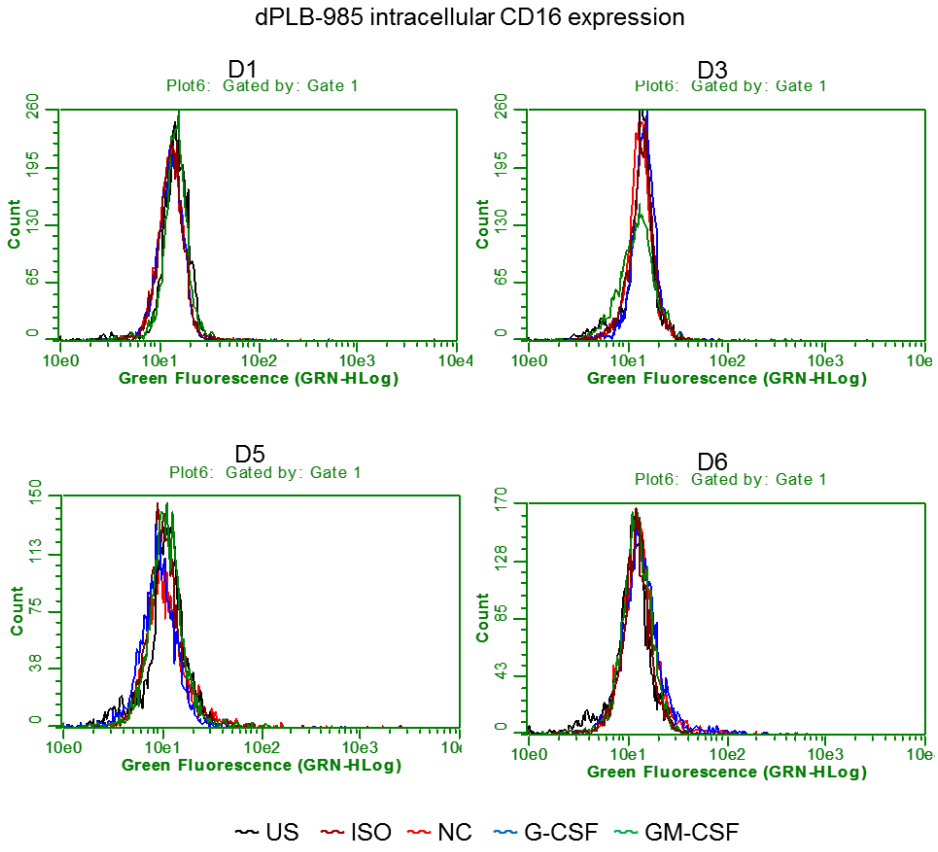


Figure 6.15 Representative flow cytometer traces of intracellular CD16 expression in dPLB-985 cells. Cells differentiated under the optimised conditions for 6 days (see Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min, were fixed by addition of equal volumes of PBS (+BSA) and PFA (4%). The cells were permeabilised with 0.1% saponin (in PBS) and stained with saturating FITC-conjugated CD16 antibody or isotype-IgG control, including unstained (US) samples (to gate for negative fluorescence). Cells were then washed with PBS (+BSA), pelleted and re-suspended in PBS (+0.1% saponin) and analysed for the expression of intracellular CD16 using flow cytometry.

6.3.11 Transmigration and chemotaxis of neutrophils

Freshly-isolated blood neutrophils incubated in the absence and presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were added into upper wells of 24-well poly-hema coated plate, separated by Millipore hanging inserts from the lower wells containing fMLP (10^{-8}M), IL-8 (0.1µg/mL), casein (0.5 mg/mL), zymosan A activated serum (ZAS) (1µg/mL) or no attractant (NA) in the lower chambers, and incubated at 37^{o}C for 90 min. Neutrophil chemotaxis was then analysed by counting the number of cells that migrated into each lower well using a Multisizer 3 cell coulter counter.

Figure 6.16 shows the transmigration of neutrophils in response to the chemotactic factors in the absence of the cytokine (NC): (fMLP 55% ± 4.36%, IL-8 27.67% ± 3.28%, casein 39.33% ± 4.41% and ZAS 27% ± 3.79%). The number of migrated cells increased after incubation with the cytokines G-CSF (fMLP 60.33% ± 4.63%, IL-8 31% ± 4.04%, casein 42.67% ± 6.01% and ZAS 29.33% ± 4.10%) or GM-CSF (fMLP 64.67% ± 4.91%, IL-8 34.33% ± 6.64%, casein 47.67% ± 2.03% and ZAS 31.67% ± 2.73%), but none of these increases reached statistical significance ($p \leq 0.05$). Neutrophils showed strong responses to all the chemotactic factors, with fMLP being the strongest.

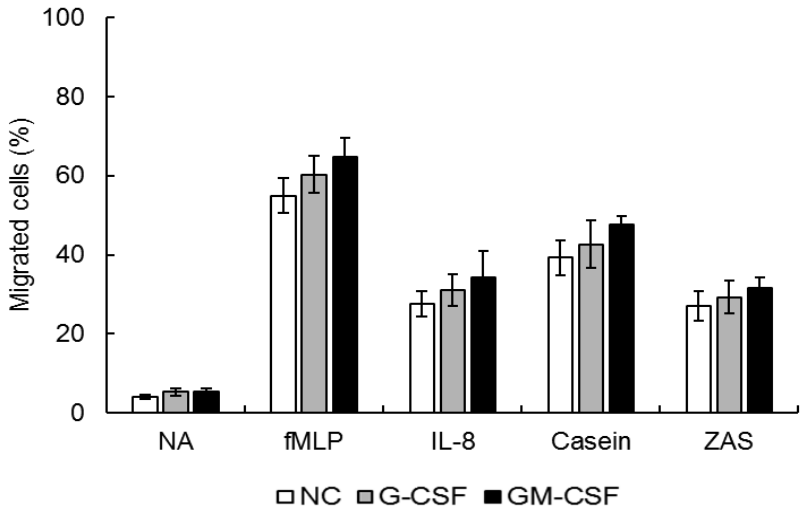


Figure 6.16 Transmigration of neutrophils towards chemotactic attractants. Freshly-isolated neutrophils incubated without (NC) and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were prepared and added into upper wells of 24-well poly-hema coated plate, separated by Millipore hanging inserts (3 µm pore sized filters) from the lower wells containing fMLP (10^{-8}M), IL-8 (0.1µg/mL), casein (0.5 mg/mL), zymosan-activated serum (1µg/mL) (ZAS contains complement factor C5a, as the active attractant) or no attractant (NA) in the lower chambers. Samples were incubated at 37^{o}C for 90 min and neutrophil chemotaxis assessed by counting the number of cells that transmigrated into each well using a Multisizer 3 cell coulter counter, following a 1:1000 dilution with Isoton II. Data are expressed as percentages of total number of cells added originally and compared to non-cytokine treated samples (± SEM, n=3).

6.3.12 Transmigration and chemotaxis of differentiated PLB-985 cells

dPLB-985 cells differentiated under the optimised conditions involving media changes and cytokine supplementation for 6 days as described in Figure 3.16, were incubated as discussed in section 6.3.10. The migration of differentiated PLB-985 cells in response to the different chemotactic factors is shown in Figure 6.17. The assay was performed on days 5 and 6 of culture and the cells displayed similar responses, with day 5 responses slightly higher. For example, at day 5 (Figure 6.17A), the percent chemotactic responses in the absence of the cytokine (NC) were: fMLP 10.33% ± 0.88%, IL-8 15.40% ± 2.03%, casein 37.33% ± 3.53% and ZAS 31.00% ± 2.08%. These responses were increased following incubation with the cytokines G-CSF or GM-CSF.

Unlike neutrophils which showed strongest chemotactic response to fMLP, differentiated PLB-985 cells showed lowest response to this formyl peptide, whereas their responses to casein and zymosan-activated serum (ZAS) were greater. This difference in chemotactic responses of neutrophils and differentiated PLB-985 cells to fMLP is consistent with that observed for chemiluminescence and oxidative burst activation as described in Chapter 5, and is likely explained by the same reason: differences in the expression of formyl peptide receptors, which may not be expressed on the differentiated PLB-985 cells. Casein and activated serum on the other hand may have attracted these cells via different receptors or via receptor-independent mechanism. Non-differentiated PLB-985 did not show chemotactic migration in response to stimulation with any of the attractants (data not shown). This further suggests that differentiation of PLB-985 into mature neutrophil-like cells triggered the observed chemotactic migration.

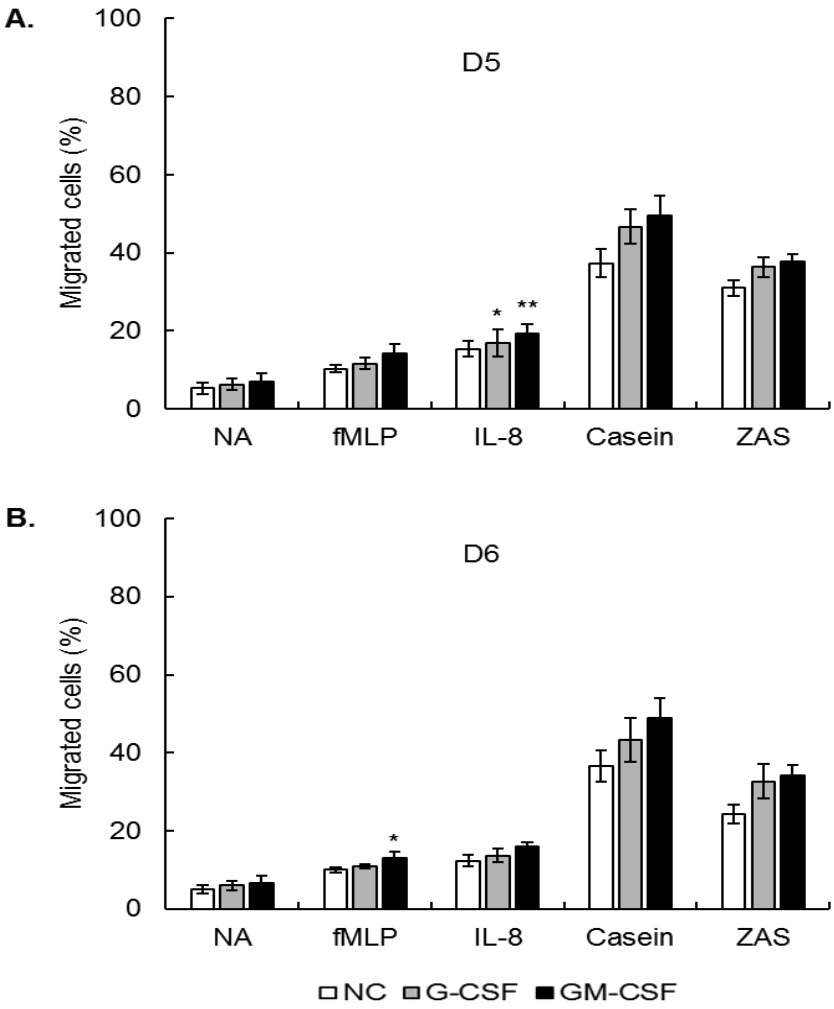


Figure 6.17 Transmigration of dPLB-985 cells towards chemotactic attractants. Cells differentiated under the optimised conditions for 6 days (see Figure 3.16) and incubated without (NC) and with the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min were, prepared and added into upper wells of 24-well poly-hema coated plates, separated by Millipore hanging inserts (3 µm pore sized filters) from the lower wells containing fMLP (10^{-8}M), IL-8 (0.1µg/mL), casein (0.5 mg/mL), ZAS (complement factor C5a) (1µg/mL) or no attractant (NA) in the lower chambers. Samples were incubated at 37°C for 90 min and dPLB-985 cells chemotaxis assessed by counting the number of cells that transmigrated into each well using a Multisizer 3 cell coulter counter, following a 1:1000 dilution with Isoton II. (A) dPLB-985 cells at day 5 of culture (B) dPLB-985 cells at day 6 of culture. Data are expressed as percentages of total number of cells added originally and compared to the NC samples (± SEM, n=3), * = p≤0.05 (paired, two-tailed student's t-test).

6.3.13 Effect of differentiation of PLB-985 cells on the activation of STAT3

dPLB-985 cells differentiated under the optimised conditions involving media changes and cytokine supplementation for 6 days as described in Figure 3.16, were incubated in the absence or presence of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) for 30 min. Protein lysates were then prepared and probed for activated STAT3 (pSTAT3), pan STAT3 and Actin protein levels by western blotting.

Figures 6.18-19 shows that pSTAT3 was not detected in dPLB-985 cells under these experimental conditions. However, pan STAT3 was detected in differentiated PLB-985 cells cultured with media changes, which slightly increased in cells incubated with G-CSF (Figure 6.18) and GM-CSF (Figure 6.19). Levels of expression of the protein decreased with increasing differentiation and the rate of decline was lower in cells incubated with cytokines than those with media changes alone. The expression of activated STAT3 (pSTAT3) in non-differentiated PLB-985 cells was equally not detected (data not shown). The activity of the pSTAT3 antibody used in these experiments was confirmed in other studies in our laboratory, in which the expression of pSTAT3 in protein extracts from isolated neutrophils and Lama 84 cells both incubated with GM-CSF for 15-30 min were detected (data not shown).

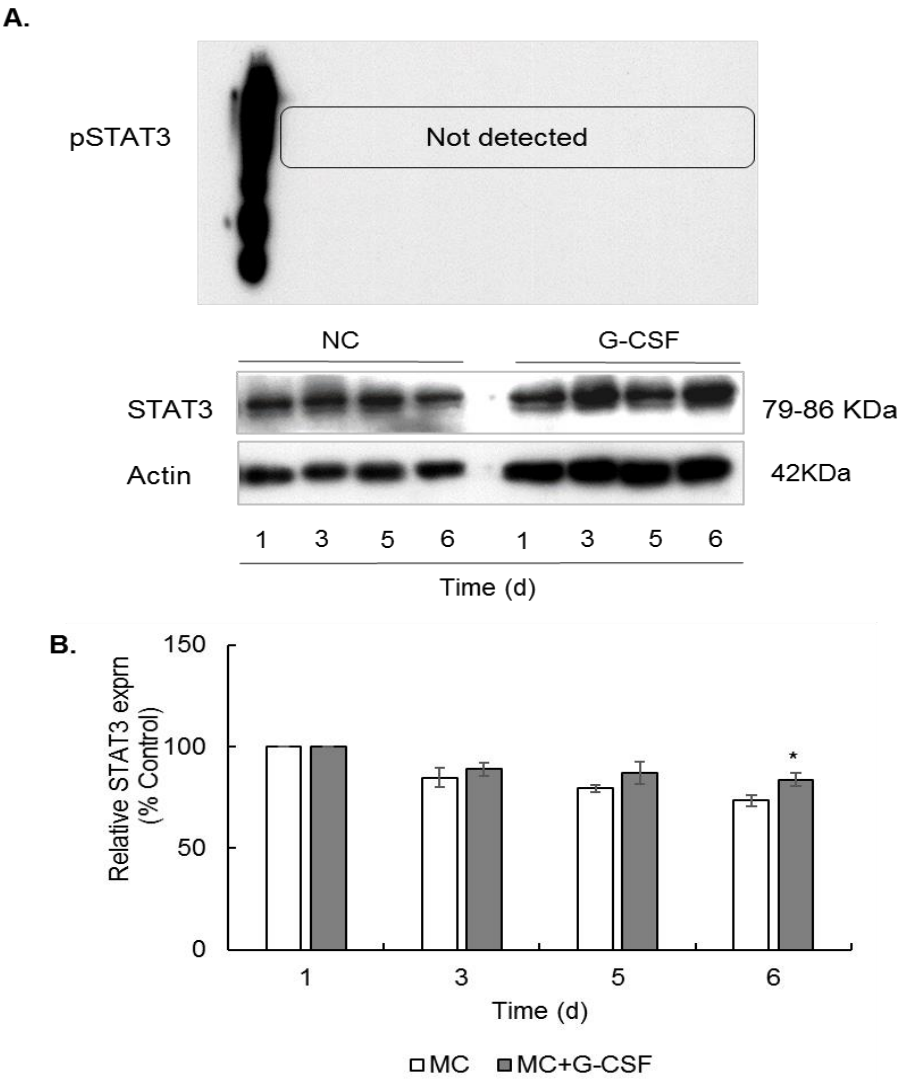


Figure 6.18 Effect of differentiation of PLB-985 cells and G-CSF on STAT3 activation. Cells were differentiated under the optimised conditions for 6 days (see Figure 3.16) and incubated in the absence (NC) and presence of the cytokines G-CSF (10ng/mL) for 30 min. Protein extracts were prepared on days 1, 3, 5, and 6 of culture and probed for activated STAT3 (pSTAT3), pan STAT3 and Actin expression levels by western blotting. (A) Western blots showing pSTAT3 (not detected), STAT3 (79-86 KDa) and Actin (42 KDa) representing three separate experiments as described above. (B) Densitometric analysis of pan STAT3 levels (normalised to the Actin signal which was the loading control). Data are expressed as percentages of day 1 taken as 100% (± SEM, n=3), * = p≤0.05 (paired, two-tailed student's t-test).

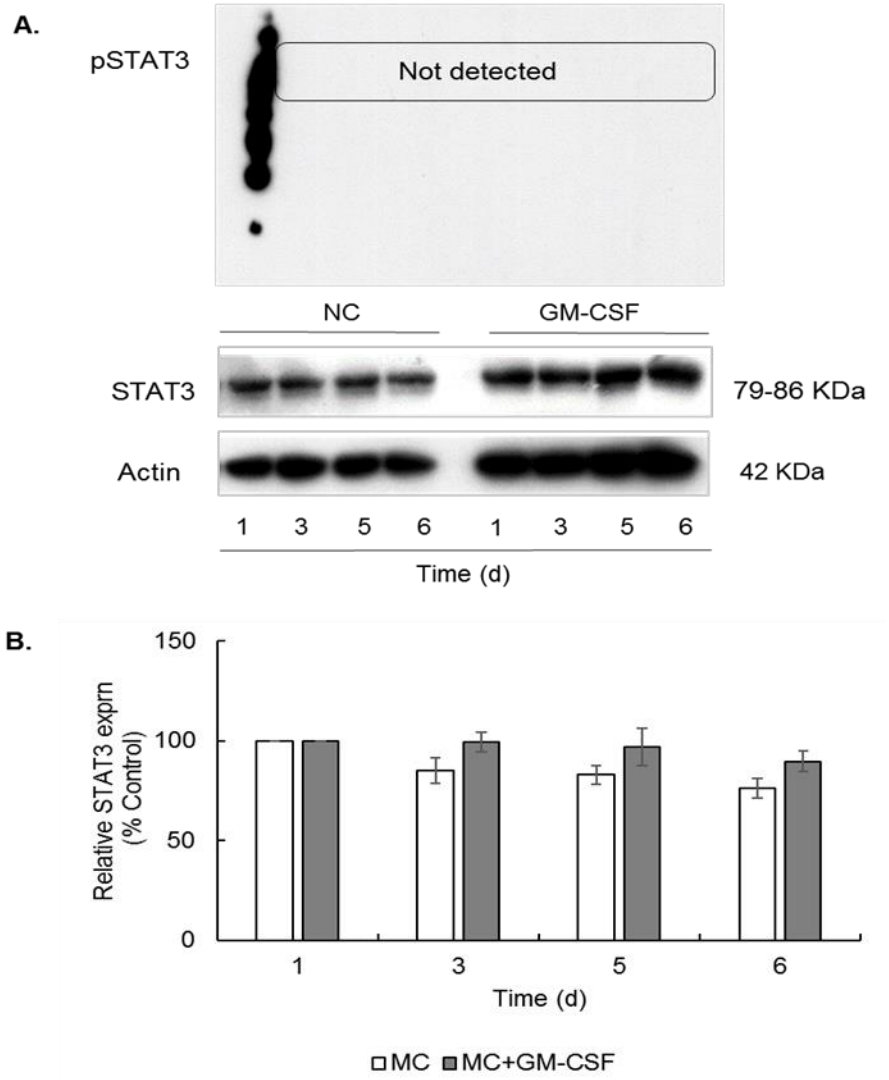


Figure 6.19 Effect of differentiation of PLB-985 cells and GM-CSF on STAT3 activation. Cells were differentiated under the optimised conditions for 6 days (as described in Figure 3.16) and incubated in the absence or presence of GM-CSF (5ng/mL) for 30 min. Protein extracts were prepared on days 1, 3, 5, and 6 of culture and probed for activated STAT3 (pSTAT3), pan STAT3 and Actin expression levels by western blotting. (A) Western blots showing pSTAT3 (not detected), STAT3 (79-86 KDa) and Actin (42 KDa) representing three separate experiments as described above. (B) Densitometric analysis of pan STAT3 levels (normalised to the Actin signal which was the loading control). Data are expressed as percentages of day 1 taken as 100% (± SEM, n=3).

6.4 Discussion and conclusions

Molecular heterogeneity of pathogens, particularly heterogeneity of their surface properties makes their recognition by neutrophils for binding and elimination, a challenging task. However, by virtue of various cell surface receptors, neutrophils recognise pathogens and other particles, which may or may not be opsonised by immunoglobulins or complement fragments. Some of these receptors recognise innate microbial structures, while others recognise opsonins generated during the inflammatory environment (e.g. complement fragments), while other receptors (e.g. FcγRs) are associated with activation of the adaptive immune response (Futosi et al., 2013). Priming of neutrophils by cytokines enhances the number and/or affinity of these receptors (Hallett & Lloyds, 1995). These receptors orchestrate the multi-step antimicrobial and inflammatory responses culminating in the clearance of invading pathogens, as well as the initiation of adaptive immune response (Parker, et al., 2005). Many of these essential components of the neutrophil cytotoxic machinery are present in the membranes of mobilizable intracellular granules which are recruited to the plasma membrane following stimulation of degranulation (Sengelov, et al., 1993).

The data presented in this Chapter have shown that CD11b (α-subunit of CR3/Mac-1) is highly expressed on both neutrophils and differentiated PLB-985 cells, with higher expression levels on neutrophils compared to differentiated PLB-985 cells. CD11b is one of the most abundant β_2-integrin on neutrophils, and is essential for their adherence to the endothelium during chemotaxis, diapedesis and phagocytosis of opsonised particles (Futosi et al., 2013). Both the isolated blood neutrophils and the neutrophil-like differentiated PLB-985 cells showed significantly increased expression of CD11b following incubations with the pro-inflammatory cytokines G-CSF and GM-CSF. This finding agrees with other published reports on up-regulation of CD11b expression on neutrophils by G-CSF and GM-CSF (Drayson, et al., 2001;

Repo, et al., 1993). Similarly, Volk *et al*., reported an increased cell surface expression of CD11b on neutrophils and differentiated cells following priming with TNF-α (Volk et al., 2011). GM-CSF was shown to up-regulate the expression of CD11b and CD18 rapidly, as well as enhance fMLP- induced ROS generation in neutrophils (Edwards, et al., 1990).

CD11b receptors are stored intracellularly in neutrophil granules from where they are recruited to replenish the cell surface levels following stimulation with inflammatory mediators or during phagocytosis (Edwards, 1994). Mobilization of intracellular stores of some proteins, such as the adhesion molecules, is one of the underlying mechanisms for the generation of a 'primed' cell phenotype, which enhances the readiness of neutrophils to respond to subsequent stimuli (Volk et al., 2011). Furthermore, some abnormalities of phagocytic functions in humans have been associated with deficiencies in adhesion proteins, such as the Mac-1/CD11b/CD18 family (Springer, et al., 1984).

The expression of myeloid cell receptor, CD14 on neutrophils and differentiated PLB-985 cells investigated in this Chapter has demonstrated that CD14 surface receptor is expressed at low levels in both cell types. This is not unexpected, because CD14 is known to be strongly expressed on monocytes and macrophages only, and at lower levels on granulocytes (Simmons, et al., 1989). However, consistent with other reports (Fleck & Nahm, 2005; Wright et al., 1991), both neutrophils and differentiated PLB-985 cells showed significantly enhanced expression of CD14 following incubations with stimulatory cytokines G-CSF and GM-CSF, but the level of expression on differentiated PLB-985 was not as high as that observed on isolated blood neutrophils.

CD14 on the surface of neutrophils serves several roles in innate immunity. It recognises LPS-opsonised particles and LPS-binding protein (LBP) and mediates the formation of LPS-LBP complex, thereby playing a role in the clearance of Gram-negative bacteria (Vosbeck et al., 1990). Also, LPS binding to CD14 causes increased activity of the adhesion receptor CD11b/CD18, promoting the neutrophils response to bacterial endotoxin, LPS (Dahinden et al., 1983). Besides its role as an innate immune receptor for 'non-self' infectious particles, CD14 also interacts with 'self' apoptotic bodies, in a receptor-ligand manner on the surface of apoptotic cells, leading to their non-inflammatory elimination (Devitt et al., 1998). In fact, CD14 serves as a recognition molecule for several other microbial constituents, in addition to LPS, including peptidoglycans, streptococcal cell wall polysaccharides, yeast cell wall protein as well as Gram-positive and Gram-negative bacteria cell wall components. In this way, CD14 potentially makes several molecules available when it binds apoptotic cells surface (Pugin et al., 1994).

The data presented on the expression of the low affinity immunoglobulin G receptor, CD16 showed that it was strongly expressed on isolated blood neutrophils, while incubation with G-CSF and GM-CSF further enhanced its expression levels. These data lend support to the reports by others (Bruhns, 2012; Perussia et al., 1983) that stimulation of neutrophils with cytokines, such as GM-CSF triggers rapid shedding of FcγRIIIb from the surface and concurrent replenishment from the internal pools. FcγRIII has two isoforms a and b, but human neutrophils constitutively express only FcγRIIIb (CD16b) and this is expressed at higher densities than all other FcγRs. CD16 is heavily glycosylated and anchored to the membrane via a glycosyl-phosphatidyl-inositol (GPI) linkage which enables its cleavage from the cell surface during shedding to provide regulation of surface expression. Hence, stimulation by cytokines, such as GM-CSF triggers rapid shedding and concurrent replenishment of FcγRIIIb to the cell surface (Bruhns, 2012; Perussia et al., 1983). GM-CSF also stimulates neutrophil

gene expression, delays apoptosis and maintains the expression of cell surface CD16 through mobilisation of intracellular stores of the pre-formed receptor (Moulding, et al., 1999).

Paradoxically, the neutrophil-like differentiated PLB-985 cells did not express appreciable amounts of surface CD16. While incubation with G-CSF and GM-CSF significantly enhanced its expression on these cells, the levels were nevertheless, very low compared to blood neutrophils. A previous study (Selmeczy, et al., 2003) however, reported expression of CD16 on DMF-differentiated PLB-985 cells by FACS analysis, whereas contrary to this, Pivot-pajot *et al.*, reported a failure to detect CD16 expression on any of the PLB-985 cells differentiated with DMSO, DMF or dbcAMP by western blot analysis (Pivot-Pajot, et al., 2010). There was also an extremely low, or zero expression of intracellular CD16 by differentiated PLB-985 cells, which could mean that there were little if any, internal stores of this receptor in these differentiated cells.

Neutrophils displayed a strong directional chemotactic transmigration towards fMLP and casein, and slightly lower chemotaxis towards IL-8 and zymosan-activated serum. Interestingly, differentiated PLB-985 cells also responded positively to all these factors. However, contrary to neutrophils which showed greater response towards fMLP, differentiated PLB-985 cells displayed a very low response towards this bacterial peptide and the cytokine IL-8, whereas their responses towards casein and zymosan activated serum were higher. The differences in chemotactic responses of neutrophils and differentiated PLB-985 cells to fMLP and IL-8 were similar to the responses observed previously in chemiluminescence and oxidative burst activation, between the two cell types described in Chapter 5, and were likely to be explained by the same reason, i.e. absence or low expression of specific receptors, on the

differentiated PLB-985 cells, as functions of these molecules are receptor-coupled (Futosi, et al., 2013; Bruhns, 2012; Migeotte et al., 2006).

Chemotactic responses of both neutrophils and differentiated PLB-985 towards all these attractants were slightly enhanced following incubations with G-CSF and GM-CSF. This observation concurs with the effects of the two colony stimulating factors (CSFs) on the regulation of various functional responses of blood neutrophils, including enhancement of differentiation, activation and survival (Barreda et al., 2004; Biethahn et al., 1999), receptor expression (Moulding et al., 1999), respiratory burst activation (Edwards et al., 1989) and chemotaxis (Wright et al., 2014; Kennedy & Deleo, 2000). Sirak *et al.,* reported that fMLP stimulated the production of hydrogen peroxide in dbcAMP differentiated HL-60 cells, but failed to induce chemotactic migration (Sirak et al., 1990). On the contrary, another study found that fMLP induced chemotaxis in DMF differentiated HL-60 cells (Fontana, et al., 1980), whilst Hauert *et al*., reported that the cytokine IL-8 was less effective in stimulating chemotaxis of differentiated HL-60, compared to neutrophils (Hauert et al., 2002). Under my experimental conditions, both fMLP and IL-8 induced chemotaxis in differentiated PLB-985 cells but fewer cells transmigrated as compared to blood neutrophils.

Chemotaxis is a special form of movement that involves temporal and spatial regulation of the cytoskeleton and intracellular signal transduction pathways, as well as re-organisation of the plasma membrane lipids. Migration of a cell towards a chemotactic attractant requires interaction of the attractant with the cell surface (Van Epps et al., 1977). Neutrophils are among the fastest moving cells in mammals, and their recruitment from the blood into the surrounding tissues, essentially involve three steps: tethering and rolling of the cells along the vascular endothelium; firm adhesion to the endothelium and finally their emigration into the tissue, through the endothelium (Kubes, 2002). To effectively clear an infection or inflammation, neutrophils must

reach the affected sites very rapidly. Upon stimulation, they can respond to both endogenous and exogenous chemotaxins, polarize and migrate directionally along the attractant's gradient. However, the mechanisms by which the cell detects the gradient, prioritize the numerous, divergent signals and migrate directionally up the gradient still remain to be completely elucidated (Nuzzi et al., 2015). Once at the site, neutrophils release antimicrobial peptides, reactive oxygen metabolites, and secrete many cytokines and chemokines, that collectively degrade and eliminate the invading microbe.

It was important to investigate the effects of differentiation of PLB-985 cells and cytokines on the activation status of signalling pathways related to the neutrophil functions. Under my experimental conditions, differentiated PLB-985 cells failed to activate STAT3 as no expression of pSTAT3 was detected. This activated protein was equally not expressed in non-differentiated PLB-985 cells, whereas pan STAT3 was detected in differentiated PLB-985 cells which was slightly raised following incubation with the cytokines G-CSF and GM-CSF. The levels of expression of this protein decreased with increasing differentiation time or as the cells aged in the culture, but the decline rate was slowed in G-CSF and GM-CSF incubated cells. Further studies are however, necessary to investigate the validity of this finding as well as the activities of other important neutrophil intracellular signalling pathways, such as STAT5, Akt, Erk and p38-MAPK in the differentiated PLB-985 cells.

In conclusion, results presented in this Chapter showed that, except for STAT3 activation, differentiated PLB-985 cells exhibited strikingly similar responses to the neutrophils in the functions investigated, such as expression of various cell surface receptors, transmigration and chemotaxis, even though the levels of expression and/or responses were lower in magnitude compared to those of isolated blood neutrophils.

Chapter 7: Effect of JAK inhibitors, Baricitinib and Tofacitinib on growth, differentiation and viability of dPLB-985 cells

7.1 Introduction

The Janus Associated Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) is a critical intracellular signal transduction pathway, downstream of many cytokine receptors in almost all multicellular organisms. Janus associated kinases are a family of tyrosine kinases that play important roles in both innate and adaptive immunity, as well as in haematopoiesis. JAK family kinases consist of four members, JAK1, JAK2, JAK3 and TYK2 (tyrosine kinase 2) and while JAK1, JAK2 and TYK2 are expressed ubiquitously, JAK3 is only expressed in haematopoietic cells (Clark et al., 2014). Nearly 40 cytokine receptors signal through combinations of the four JAKs and seven STATs, indicating common features across the JAK-STAT signalling cascade (Murray, 2007).

Cytokines regulate many important cellular functions related to haematopoiesis, proliferation, differentiation, maturation and apoptosis (Wu & Sun, 2012). Several cytokine receptors signal through the JAK-STAT and the MAP Kinase pathways (Murray, 2007). Inflammatory cytokine receptors signal through JAK1 and JAK2, and conversely, several growth factor receptors required for myeloid and erythroid haematopoiesis signal through JAK3 (Meyer et al., 2010). In resting cells, JAKs interact with subcellular domains of type I and type II cytokine receptors, and upon binding of a cytokine to its cognate receptor, JAKs are activated, which in turn activates STAT proteins by tyrosine phosphorylation, leading to a rapid signalling from the plasma membrane to the nucleus, where the activated transcription factors modulate the expression of target genes (see Figure 1.9) (Meyer et al., 2010;

Vijayakrishnan et al., 2010). Aberrant expression of cytokines and growth factors has been implicated in the pathogenesis of autoimmune and chronic inflammatory diseases (Imada & Leonard, 2000).

Biologic drugs targeting specific cytokines and their receptors, such as TNF-α inhibitors have been developed and have proven effective in the treatment of chronic inflammatory diseases, including rheumatoid arthritis (Maini & Taylor, 2000). However, factors such as emerging resistance, increased rates of infection, high costs of treatment, as well as injection-related complications, have led to the search for safer drugs that can selectively interfere with molecular mediators of cytokine signalling and whose actions are limited to immune cells (Pesu et al., 2008). In this context, several orally-active, low molecular weight (<800 Da) JAK inhibitors that target specifically the disease-associated molecules and cytokine receptor signalling pathways are being developed as therapeutic agents (Meyer et al., 2010). Currently, there are at least 10 different JAK inhibitors at various phases of clinical trials (Furqan et al., 2013).

Baricitinib (LY-3009104) and Tofacitinib (CP-690550) are among the JAK inhibitors in advanced stages of clinical trials, for the treatment of haematopoietic disorders, transplant rejection as well as autoimmune and inflammatory diseases (Miossec, 2013). Baricitinib is a potent and selective inhibitor of JAK1 and JAK2 enzymes (Shi et al., 2014) while Tofacitinib is a novel and specific inhibitor of JAK3 and JAK1 (JAK3 forms a complex with JAK1, so JAK3 inhibition affects JAK1). Tofacitinib was initially developed as a selective JAK3 inhibitor, but recent studies in cell culture have shown that it suppressed JAK1/3, JAK1/2, and JAK1/TYK2 cytokine signalling, but with much lower activity against JAK2. Many of these effects on different JAKs are however, concentration dependent (Miossec, 2013, Ghoreschi et al., 2011).

JAK-STAT signalling enhances the expression of many inflammatory cytokines and chemokines. This suggests that JAK inhibitors may inhibit production of cytokines and chemokines (Hu & Ivashkiv, 2009), which partly explains their efficacy in the treatment of inflammatory diseases (Yarilina et al., 2012). Thus, it would be worthwhile to investigate the effects of these small molecule JAK inhibitors on proliferation, differentiation and survival of differentiated PLB-985 cells, with focus on the proinflammatory cytokines G-CSF and GM-CSF, because of their pivotal roles in differentiation and survival of granulocytes.

The aims of the work in this Chapter therefore were:

1. To determine the effect of JAK inhibitors, baricitinib and tofacitinib on the growth, differentiation and viability of dPLB-985 cells cultured without and with differentiation media changes.
2. To determine the effect of these JAK inhibitors on growth, differentiation and viability of dPLB-985 cells in presence of the cytokines, G-CSF and GM-CSF.

7.2 Methods

dPLB-985 cells were differentiated under the different conditions described in Chapter 3. The cells were incubated in the absence and presence of JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min followed by addition of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL) and the cells were assayed for the following functions.

i. **Cell growth:** Cell numbers were measured using the Multisizer 3 coulter cell counter, following a 1:1000 dilution with Isoton II.

ii. **Cell viability:** Cell's viability was analysed using the Guava viaCount assay on the flow cytometer.

iii. **Cell morphology:** Differentiation and apoptosis were determined via cell morphology examination. Following different incubations, cytospin slides of cells samples were prepared and stained with rapid Romanowsky stains. Cell morphology was then viewed under a light microscope. Cells images were captured using a microscope-mounted camera and percent differentiated or apoptotic cells determined manually by differential cell counting.

7.3 Results

7.3.1 Effects of Jak inhibitors on growth of dPLB-985 cells

Cells at 4×10^5/mL were incubated without (NI) and with JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) in differentiation media for 7 days, without and with media changes and inhibitor replenishment on days 2 and 4, and the cell numbers were counted using the Multisizer 3 coulter cell counter.

Figure 7.1 shows the growth curve of dPLB-985 cells cultured under these conditions. Uninhibited cells cultured without media changes (Figure 7.1A) grew exponentially, reaching a maximum population density on day 4: $2.44\times10^6 \pm 5.87\times10^4$ and then numbers declined over the following 3 days of culture. In the presence of baricitinib, cell numbers were consistently lower than in the absence of this inhibitor, while tofacitinib further decreased the cell counts. Cells cultured with media changes on days 2 and 4 (Figure 7.1B) showed enhanced exponential growth, with maximum

density on day 5: $2.60\times10^6 \pm 9.56\times10^4$, which then declined during culture over the following 3 days. In the presence of baricitinib, cell numbers were decreased significantly ($p\leq0.05$) and a maximum cell density at day 5 was $2.24\times10^6 \pm 5.73\times10^4$. Similarly, in the presence of tofacitinib, cell numbers were decreased at day 5 to $2.02\times10^6 \pm 8.00\times10^4$. Hence, while the media changes significantly enhanced the growth rate of dPLB-985 cells compared to no media changes, this enhanced growth was downregulated by inhibition with baricitinib and tofacitinib, with the latter showing a more inhibitory effect.

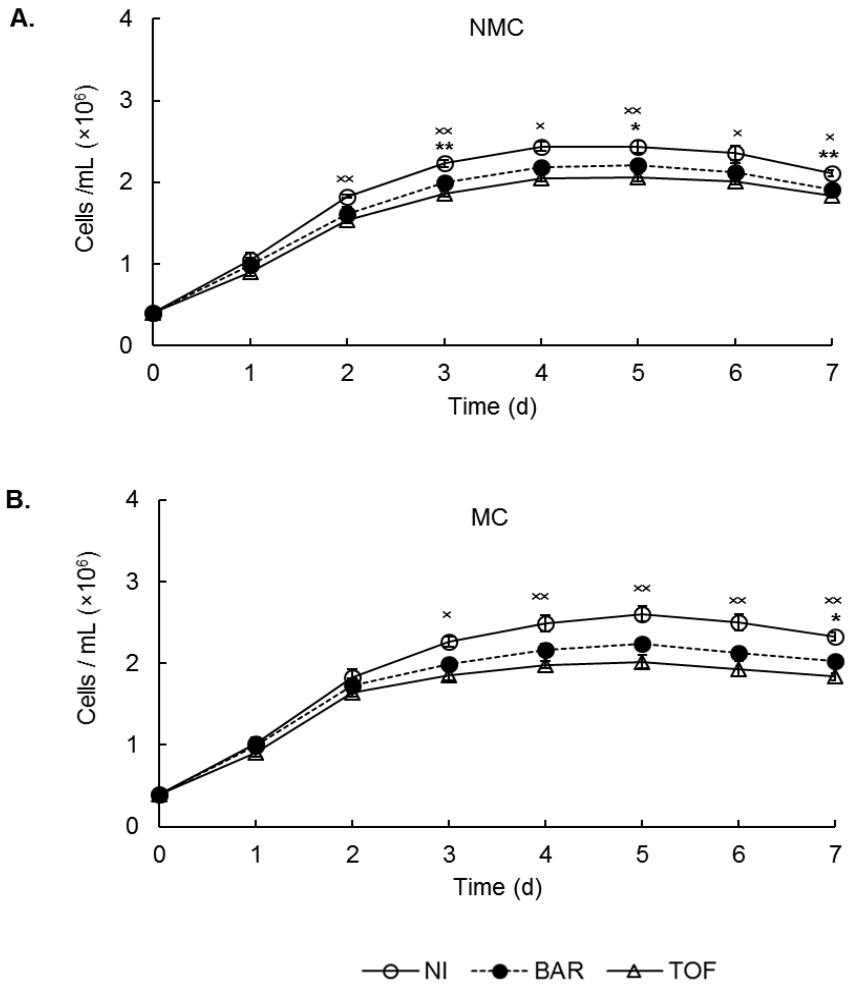


Figure 7.1 Effect of JAK inhibitors on the growth of differentiated PLB-985 cells. Exponentially growing PLB-985 cells at a starting density of 4×10^5/mL were incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) in differentiation media. The cultures were incubated for 7 days without (A, NMC) and with media changes (B, MC) and inhibitor replenishments on days 2 and 4 of culture, and cell numbers counted daily using the multisizer 3 coulter counter following a 1:1000 dilution with Isoton II. Data are expressed as mean of total cell number (± SEM, n=3), * = p≤0.05, ** = p≤0.01, * and ˣ indicate significant levels of baricitinib and tofacitinib respectively, compared to cell numbers of uninhibited cells at the same time point (paired, two-tailed student's t-test).

7.3.2 Effects of Jak inhibitors on growth of dPLB-985 cells in the presence of cytokines

Cells seeded at 4×10^5/mL were incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min, followed by addition of the cytokines G-CSF (10ng/mL) or GM-CSF (5 ng/mL). The cultures were incubated for 7 days with differentiation media changes and replenishment of inhibitors and cytokines on days 2 and 4, and the cell numbers were counted using the Multisizer 3 coulter cell counter.

Growth of dPLB-985 cells incubated with JAK inhibitors and cytokines is shown in Figure 7.2. Uninhibited cells (NI) incubated with G-CSF (Figure 7.2A) exhibited a maximum population density on day 5: $2.85\times10^6 \pm 8.33\times10^4$, which declined over days 6 and 7. This is a markedly high growth compared to uninhibited cells with differentiation media changes alone (MC, Figure 7.1B). However, these cell numbers significantly decreased following incubation with baricitinib, which showed cell density of $2.41\times10^6 \pm 8.50\times10^4$ at day 5. Uninhibited cells (NI) incubated with GM-CSF (Figure 7.2B) showed enhanced cell number, with maximum growth of $3.26\times10^6 \pm 6.49\times10^4$ on day 5, also compared to uninhibited cultures with differentiation media changes alone (MC, Figure 7.1B). Again, incubation with baricitinib significantly ($p\leq0.05$) decreased the growth: at day 5 the cell density was $2.35\times10^6 \pm 7.83\times10^4$. Similarly, tofacitinib inhibited GM-CSF enhanced cell growth: day 5 cell density of $2.06\times10^6 \pm 4.91\times10^4$. Both cytokines therefore, significantly enhanced the growth of dPLB-985 cells with GM-CSF showing stronger effect. However, these growth-stimulating effects of the cytokines were downregulated significantly by both inhibitors, with tofacitinib showing greater effects.

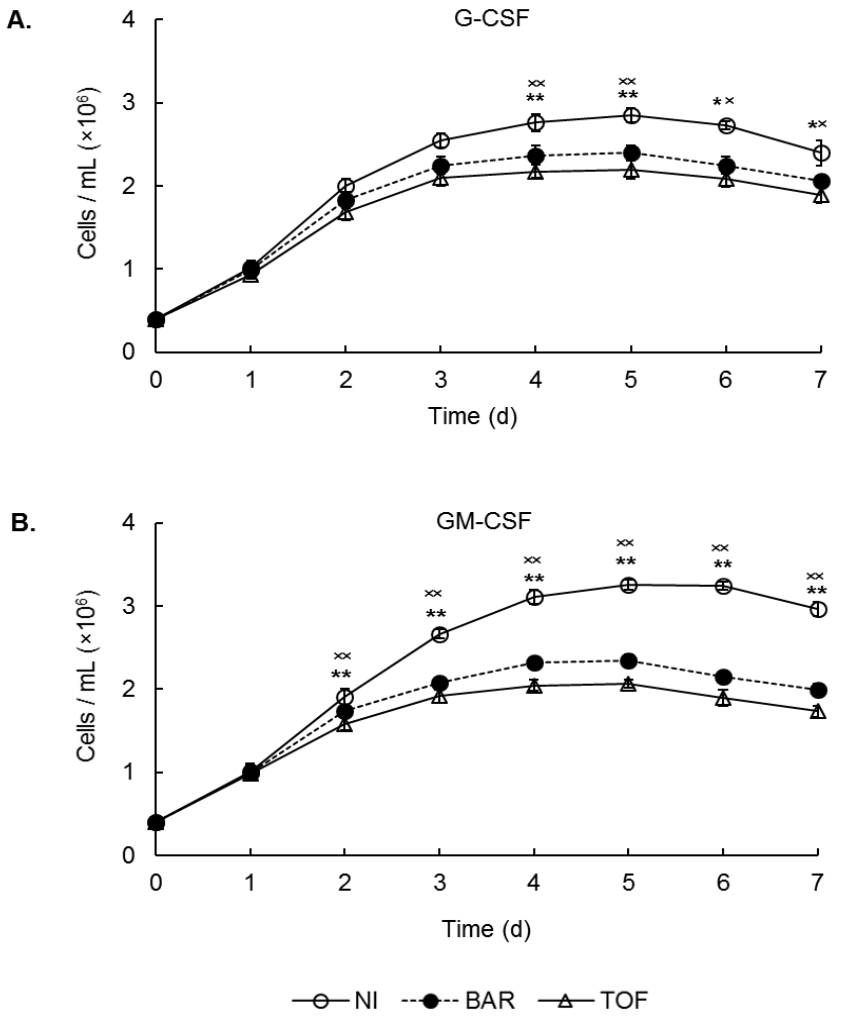


Figure 7.2 Effect of JAK inhibitors on growth of dPLB-985 cells in the presence of cytokines. Exponentially-growing PLB-985 cells at a starting density of 4×10^5/mL were incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min, followed by the addition of the cytokines G-CSF (A,10ng/mL) or GM-CSF (B, 5ng/mL) and incubated for 7 days. Fresh differentiation media changes and replenishment of inhibitors and cytokines were made on days 2 and 4. Cell numbers were counted daily using the multisizer 3 coulter counter, following a 1:1000 dilution with Isoton II. Data are expressed as mean of total cell number (± SEM, n=3), * = p≤0.05, ** = p≤0.01, * and × indicate significant levels of baricitinib and tofacitinib respectively, compared to cell numbers of uninhibited cells at the same time point (paired, two-tailed student's t-test).

7.3.3 Effects of Jak inhibitors on the viability of dPLB-985 cells

Cells were seeded at 4×10^5/mL and incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) in differentiation media for 7 days, without and with media changes and inhibitor replenishments on days 2 and 4. Cells viability was measured using the Guava viaCount assay by flow cytometry. The initial viability of PLB-985 cells at day 0 before induction of differentiation was 97.10% ± 0.30%. In cells cultured with unchanged differentiation media (NMC: Figure 7.3A), this viability declined progressively over the incubation period, being 60.11% ± 2.91% at day 5 and 26.97% ± 0.60% at day 7.

There was also significant ($p\leq0.05$) decrease in viability following incubation with baricitinib, for example 53.45% ± 3.69% at day 5 and 21.97% ± 2.29% at day 7. These effects on viability were even more pronounced after incubation with tofacitinib: 48.45% ± 1.32% at day 5 and 20.30% ± 2.82% at day 7. Whereas in cells cultured with media changes (MC: Figure 7.3B), the viability also declined: 68.82% ± 2.31% viability at day 5 and 43.60% ± 2.94% at day 7, but this was higher than culture with unchanged media. There was also a significant ($p\leq0.05$) progressive decrease in this viability following incubation with baricitinib: 63.50% ± 1.60% at day 5 and 36.92% ± 2.86% at day 7, and with tofacitinib: 60.16% ± 1.93% at day 5 34.92% ± 2.41% at day 7. Therefore, the viability of dPLB-985 cells was markedly enhanced when the differentiation media was replaced at day 2 and 4, but the JAK inhibitors, baricitinib and tofacitinib reversed this increase in viability.

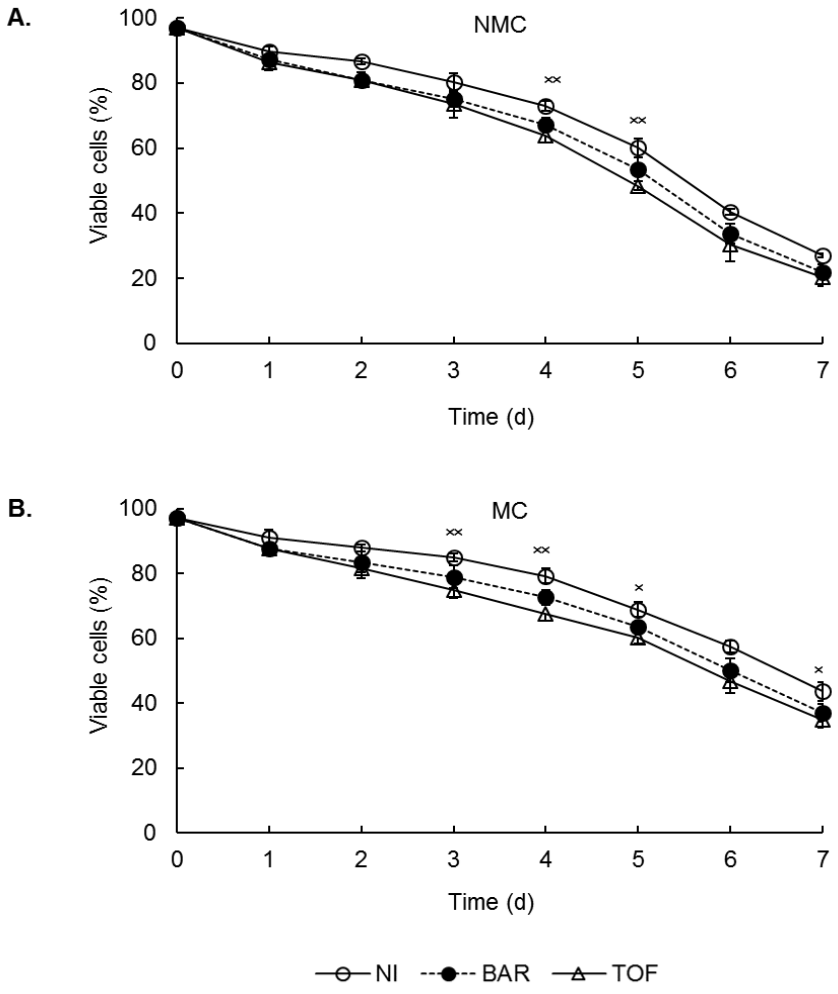


Figure 7.3 Effect of JAK inhibitors on viability of differentiated PLB-985 cells. Exponentially-growing dPLB-985 cells at a starting density of 4×10^5/mL were incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL), for 7 days without (A, NMC) and with fresh media changes (B, MC) and inhibitor replenishment on days 2 and 4. Cell viability was measured using the Guava viaCount assay by flow cytometry. Data are expressed as mean of percent total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01, * and ˣ indicate significant levels of baricitinib and tofacitinib respectively, compared to cell numbers of uninhibited cells at the same time point (paired, two-tailed student's t-test).

7.3.4 Effects of Jak inhibitors on viability of dPLB-985 cells in the presence of cytokines

Cells seeded at 4×10^5/mL were incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min, followed by addition of the cytokines G-CSF (10ng/mL) or GM-CSF (5 ng/mL). The cultures were incubated for 7 days with differentiation media changes and replenishment of inhibitors and cytokines on days 2 and 4, and cell viability was measured using the Guava viaCount assay by flow cytometry.

Cell viability under these experimental conditions are shown in Figure 7.4. The initial viability at the cells at d 0 was 97.03% ± 0.23%. In uninhibited cells (NI) incubated with the G-CSF (Figure 7.4A), the cell viability declined during culture: 80.75 ± 0.13% at day 5 and 52.88% ± 2.18% at day 7. Therefore, incubation with G-CSF enhanced the survival of dPLB-985 cells compared to media changes alone (MC, Figure 7.3B). However, in the presence of JAK inhibitors, this viability significantly ($p\leq0.05$) decreased. For example, with baricitinib viability was 72.87% ± 1.13% at day 5 and 44.81% ± 2.34% at day 7, and with tofacitinib viability was 67.02% ± 1.90% at day 5 and 43.16% ± 1.65% at day 7.

For GM-CSF incubated cultures (Figure 7.4B), the viability of cells in the absence of JAK inhibitors (NI) declined slightly over culture period. For example, 86.77% ± 2.86% at day 5 and 68.20% ± 1.43% at day 7, which was greater viability compared to cultures containing no cytokine (MC, Figure 7.3B). Following incubation with inhibitors, the viability decreased significantly ($p\leq0.05$) with baricitinib: 74.90% ± 2.32% at day 5 and 54.85% ± 0.57% at day 7, and with tofacitinib: 70.24% ± 1.64% at day 5 and 48.80% ± 1.30% at day 7. The cytokines G-CSF and GM-CSF promoted the survival of dPLB-985 cells considerably, but their effect was also significantly

downregulated by both JAK inhibitors. GM-CSF was more potent than G-CSF in promoting viability and tofacitinib was more potent than baricitinib in decreasing the viability.

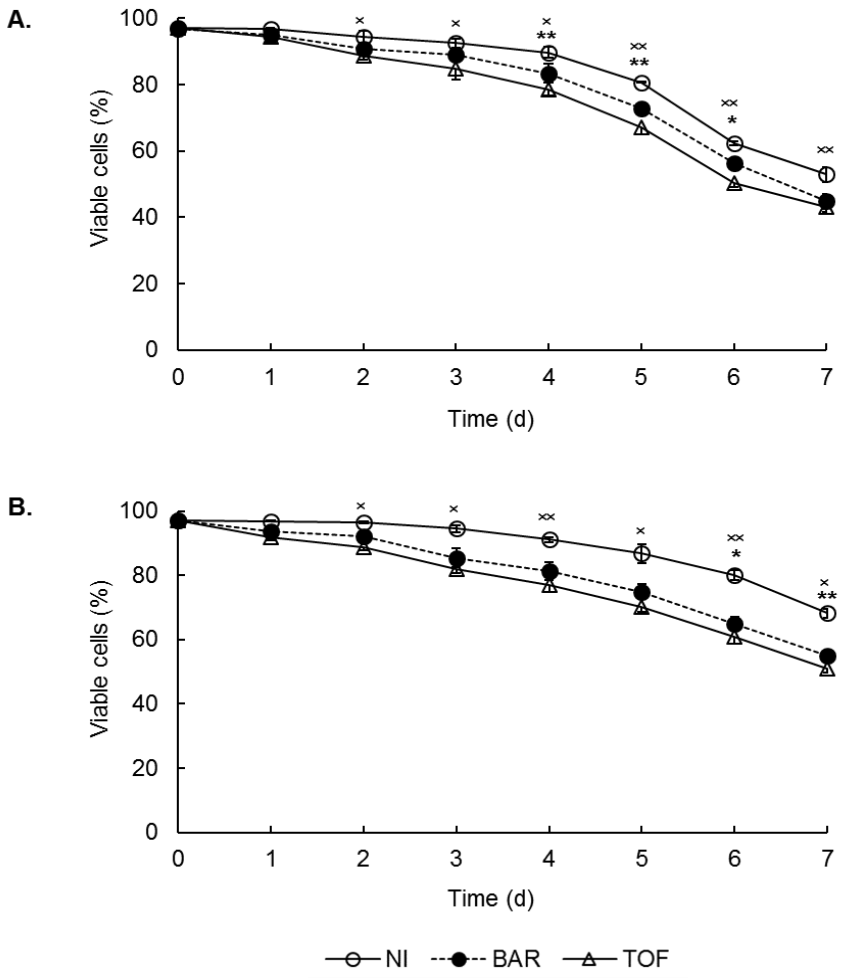


Figure 7.4 Effect of JAK inhibitors on viability of dPLB-985 cells in the presence of cytokines. Exponentially-growing PLB-985 cells at a starting density of 4×10^5/mL were incubated without (NI) and with JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min, followed by the addition of the cytokines G-CSF (A,10ng/mL) or GM-CSF (B, 5ng/mL) and incubated for 7 days. Fresh differentiation media changes and replenishment of inhibitors and cytokines were done after day 2 and 4. Cell viability was measured using the Guava viaCount assay by flow cytometry. Data are expressed as mean of percent total cells (± SEM, n=3), * = p≤0.05, ** = p≤0.01, * and × indicate significant levels of baricitinib and tofacitinib respectively, compared to cell numbers of uninhibited cells at the same time point (paired, two-tailed student's t-test).

7.3.5 Effects of Jak inhibitors on differentiation of PLB-985 cells

Cells seeded at 4×10^5/mL were incubated without (NI) and with JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) in differentiation media. The cultures were incubated for 7 days without and with media changes and inhibitor replenishment on days 2 and 4. Cytospin slides of differentiated PLB-985 cells were prepared daily, stained with rapid Romanowsky stains, and percent differentiated cells was determined by differential counting, manually under a light microscope.

Figure 7.5 shows the percent differentiation of dPLB-985 cells. For cultures with no media changes (NMC, Figure 7.5A), the percent differentiation in the absence of inhibitors (NI) was 51.23% ± 3.11% at day 4 and 54.37% ± 5.01% at day 6, which decreased during culture with JAK inhibitors, baricitinib: 47.91% ± 1.68% at day 4 and 54.05% ± 2.99% at day 6, and tofacitinib: 44.95% ± 2.90% at day 4 and 51.80% ± 4.97% at day 6, but this decrease did not reach statistical significance. In cultures with media changes (MC, Figure 7.5B), percent differentiated cells in the absence of inhibitors (NI) was 56.90% ± 3.14% at day 4 and 65.04% ± 3.01% at day 6, indicating an increased differentiation compared to no media changes (NMC, Figure 7.5A). However, percent differentiation decreased during culture with the JAK inhibitors. For example, with baricitinib: 51.91% ± 4.93% at day 4 and 60.05% ± 3.40% at day 6, and with tofacitinib: 48.29% ± 3.96% at day 4 and 58.47% ± 2.42% at day 6, but these decreases did not reach statistical significance. Therefore, while media changes enhanced the differentiation of dPLB-985 cells, this was insignificantly decreased by the JAK inhibitors, baricitinib and tofacitinb. Representative cytospin slides images of dPLB-985 cells differentiated under these experimental conditions are shown in Figure 7.9.

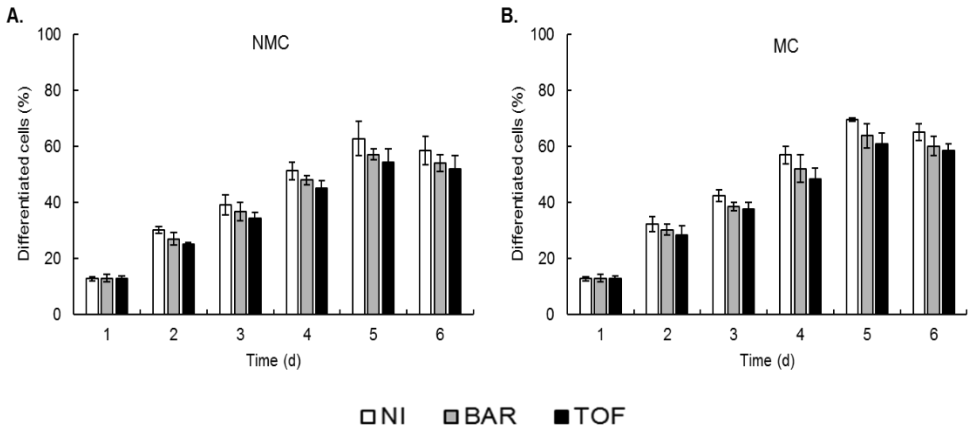


Figure 7.5 Effect of JAK inhibitors on differentiation of PLB-985 cells. Exponentially-growing PLB-985 cells at a starting density of 4×10^5/mL were incubated without (NI) and with JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 6 days without (A, NMC) and with media changes (B, MC) and inhibitors replenishment on days 2 and 4. Cytospin slides of cells were prepared, stained with rapid Romanowsky stain daily, and percent differentiated cells determined by differential counting manually under the light microscope. Data are expressed as mean of percent total cells (± SEM, n=3), and compared to cell numbers of uninhibited cells at the same time point.

7.3.6 Effects of Jak inhibitors on differentiation of PLB-985 cells in the presence of cytokines

Cells seeded at 4×10^5/mL were incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min, followed by addition of the cytokines G-CSF (10ng/mL) or GM-CSF (5 ng/mL). The cultures were incubated for 7 days with differentiation media changes and replenishment of inhibitors and cytokines on days 2 and 4. Cytospin slides of differentiated PLB-985 cells were prepared daily, stained with rapid Romanowsky stain, and percent differentiated cells was determined by differential counting manually, under the light microscope. As shown in Figure 7.6, the percent differentiation of cells incubated with G-CSF in the absence (NI) of JAK inhibitors (Figure 7.6A) was 67.21% ± 1.76% at day 4 and 76.51% ± 2.11% at day 6, but this decreased significantly ($p\leq0.05$) following incubation with baricitinib: 59.45% ± 2.97% at day 4 and 70.35% ± 2.12% at day 6 and with tofacitinib: 54.36% ± 3.35% at day 4 and 66.40% ± 2.26% at day 6. In GM-CSF incubated cultures (Figure 7.6B), the percent differentiated cells in the absence of JAK inhibitors (NI) was higher than that observed with G-CSF. For example, 71.70% ± 1.33% on day 4 and 77.53% ± 2.53% on day 6, which also decreased significantly ($p\leq0.05$) following incubation with baricitinib: 63.33% ± 3.24% at day 4 and 70.37% ± 2.17% at day 6, and with tofacitinib: 57.97% ± 4.52% at day 4 and 66.67% ± 3.30% at day 6.

It is therefore, evident from these data that incubation with both G-CSF and GM-CSF substantially enhanced the differentiation of PLB-985 cells, compared to media changes alone. However, these effects were down-regulated by incubations with baricitinib and tofacitinib. Representative cytospin slides images of dPLB-985 cells differentiated under these experimental conditions are shown in Figures 7.10 and 7.11.

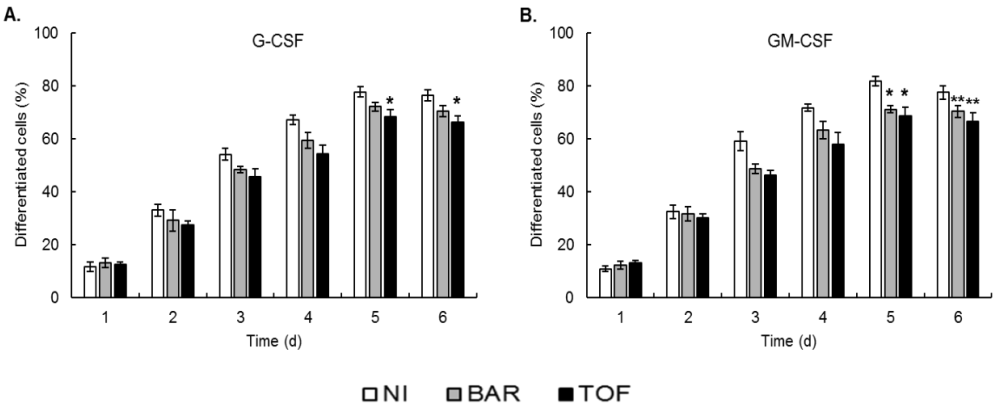


Figure 7.6 Effect of JAK inhibitors on differentiation of PLB-985 cells in the presence of cytokines. Exponentially-growing PLB-985 cells at a starting density of 4×10^5/mL were incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min, followed by addition of the cytokines G-CSF (A, 10ng/mL) or GM-CSF (B, 5ng/mL) and incubated for 6 days. Fresh differentiation media changes and replenishment of inhibitors and cytokines were done on days 2 and 4. Cytospin slides of cells were prepared, stained with rapid Romanowsky stain daily, and percent differentiated cells determined by differential counting manually under the light microscope. Data are expressed as mean of percent total cells (± SEM, n=3), and compared to cell numbers of uninhibited cells at the same time point, * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

7.3.7 Effects of Jak inhibitors on apoptosis of dPLB-985 cells

Cells seeded at 4×10^5/mL were incubated without (NI) and with JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) in differentiation media. The cultures were incubated for 6 days without and with media changes and inhibitor replenishment on days 2 and 4. Cytospin slides of cells samples were prepared daily, stained with rapid Romanowsky stain, and percent apoptotic cells was determined by differential counting manually under a light microscope.

Figure 7.7 shows the apoptosis of dPLB-985 cells under these conditions. For cultures with unchanged media (NMC, Figure 7.7A), percent apoptosis in the absence of inhibitors (NI) was 32.00% ± 1.73% at day 4 and 38.73% ± 2.05% at day 6. Following incubation with JAK inhibitors, apoptosis increased significantly ($p\leq0.05$). For example, with baricitinib: 38.00% ± 2.08% at day 4 and 42.70% ± 2.26% at day 6, and with tofacitinib: 41.74% ± 0.70% at day 4 and 45.17% ± 1.92% at day 6. Whereas in cultures with media changes (MC, Figure 7.7B), percent apoptosis in the absence of the inhibitors (NI) was: 28.67% ± 2.03% at day 4 and 35.40% ± 1.76% at day 6. Although this was lower than the percent apoptosis observed in cultures with no media changes (NMC, Figure 7.7A), again the apoptosis markedly increased following incubation with JAK inhibitors, baricitinib: 34.67% ± 2.33% at day 4 and 39.37% ± 1.37% at day 6, and tofacitinib: 38.41% ± 3.28% at day 4 and 41.83% ± 2.32% at day 6.

This data clearly indicates that differentiation media changes decrease the apoptosis of dPLB-985 cells, thereby promoting their survival, but the JAK inhibitors, baricitinib and tofacitinib decreased this protective effect of the media changes. Representative cytospin slides images of apoptotic dPLB-985 cells under these experimental conditions are shown in Figure 7.9.

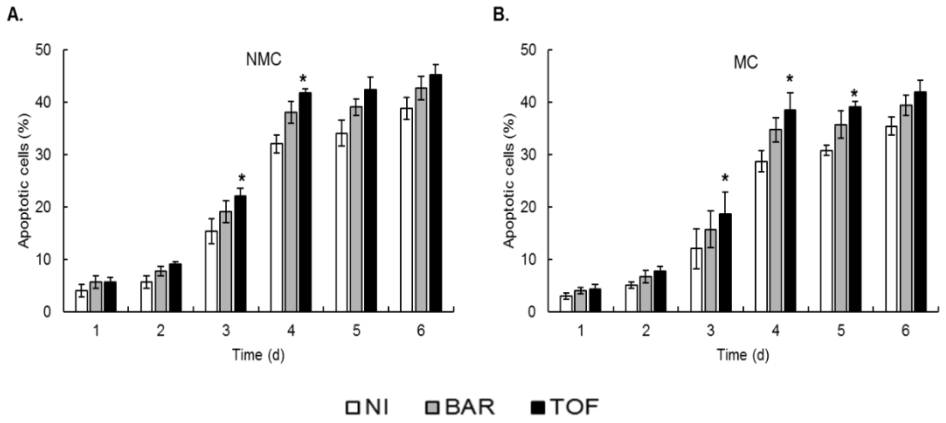


Figure 7.7 Effects of JAK inhibitors on apoptosis of differentiated PLB-985 cell. Exponentially-growing PLB-985 cells at a starting density of 4×10^5/mL were incubated without (NI) and with the JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) in differentiation media, and the culture incubated for 6 days without (A, NMC) and with media changes (B, MC) and inhibitor replenishment on days 2 and 4. Cytospin slides of cells samples were prepared, stained with rapid Romanowsky stain daily, and percent apoptotic cells determined by differential counting manually under the light microscope. Data are expressed as mean of percent total cells (± SEM, n=3) and compared to cell numbers of uninhibited cells at the same time point, * = p≤0.05, (paired, two-tailed student's t-test).

7.3.8 Effects of Jak inhibitors on apoptosis of dPLB-985 cells in the presence of cytokines

Cells seeded at 4×10^5/mL were incubated without (NI) and with JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min, followed by addition of the cytokines G-CSF (10ng/mL) or GM-CSF (5ng/mL). The cultures were incubated for 6 days with differentiation media changes and replenishment of inhibitors and cytokines additions on days 2 and 4. Cytospin slides of cells samples were prepared daily, stained with rapid Romanowsky stain, and percent apoptotic cells was determined by differential counting manually under a light microscope.

For G-CSF incubated cultures (Figure 7.8A), the percent apoptosis in the absence of inhibitor (NI) was 21.37% ± 1.89% at day 4 and 30.10% ± 1.88% at day 6, which were lower than percent apoptosis observed in cells with media changes alone (MC, 7.7B). However, the apoptosis levels were higher in cultures containing the JAK inhibitors. For example, with baricitinib: 26.79% ± 1.98% at day 4 and 35.97% ± 1.39% at day 6, and with tofacitinib: 28.57% ± 2.08% at day 4 and 38.30% ± 1.65% at day 6. Similarly, in GM-CSF incubated cultures (Figure 7.8B), the percent apoptosis in the absence of inhibitor (NI) was 21.13% ± 1.92% at day 4 and 29.57% ± 1.94% at day 6, which were also significantly lower than the apoptosis levels observed in cells with media changes alone (MC, 7.7B). Apoptosis was significantly increased by incubation with JAK inhibitors, baricitinib: 25.62% ± 2.36% at day 4 and 34.00% ± 1.91% at day 6, and tofacitinib: 27.40% ± 2.42% at day 4 and 36.53% ± 1.36% at day 6.

These data suggest that cytokines G-CSF and GM-CSF significantly delayed apoptosis of dPLB-985 cells, thereby prolonging their survival, with the effect of GM-CSF being higher than that of G-CSF. Also, the JAK inhibitors baricitinib and tofacitinib significantly down-regulated the effects of these cytokines, thereby

increasing the apoptosis of dPLB-985 cells. Representative cytospin slides images of apoptotic dPLB-985 cells under these experimental conditions are shown in Figures 7.10 and 7.11.

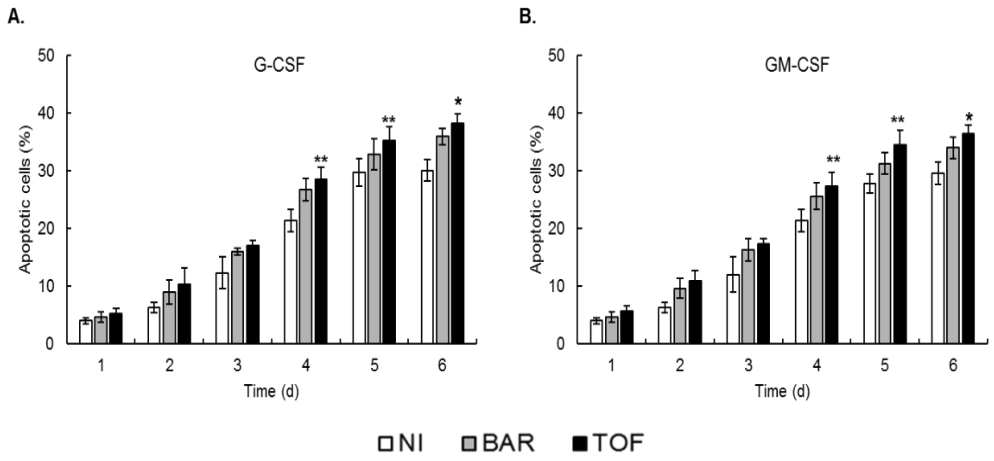


Figure 7.8 Effect of JAK inhibitors on apoptosis of dPLB-985 cells in the presence of cytokine. Exponentially-growing PLB-985 cells at a starting density of 4×10^5/mL were seeded in differentiation media without (NI) and with JAK inhibitors baricitinib (200ng/mL) or tofacitinib (200ng/mL) for 30 min, followed by the addition of the cytokines G-CSF (A, 10ng/mL) or GM-CSF (B, 5ng/mL) and incubated for 6 days. Fresh differentiation media changes and replenishment of inhibitors and cytokines were made on days 2 and 4. Cytospin slides of cells were prepared, stained with rapid Romanowsky stain daily, and percent apoptotic cells determined by differential counting manually under the light microscope. Data are expressed as mean of percent total cells (± SEM, n=3) and compared to cell numbers of uninhibited cells at the same time point, * = p≤0.05, ** = p≤0.01 (paired, two-tailed student's t-test).

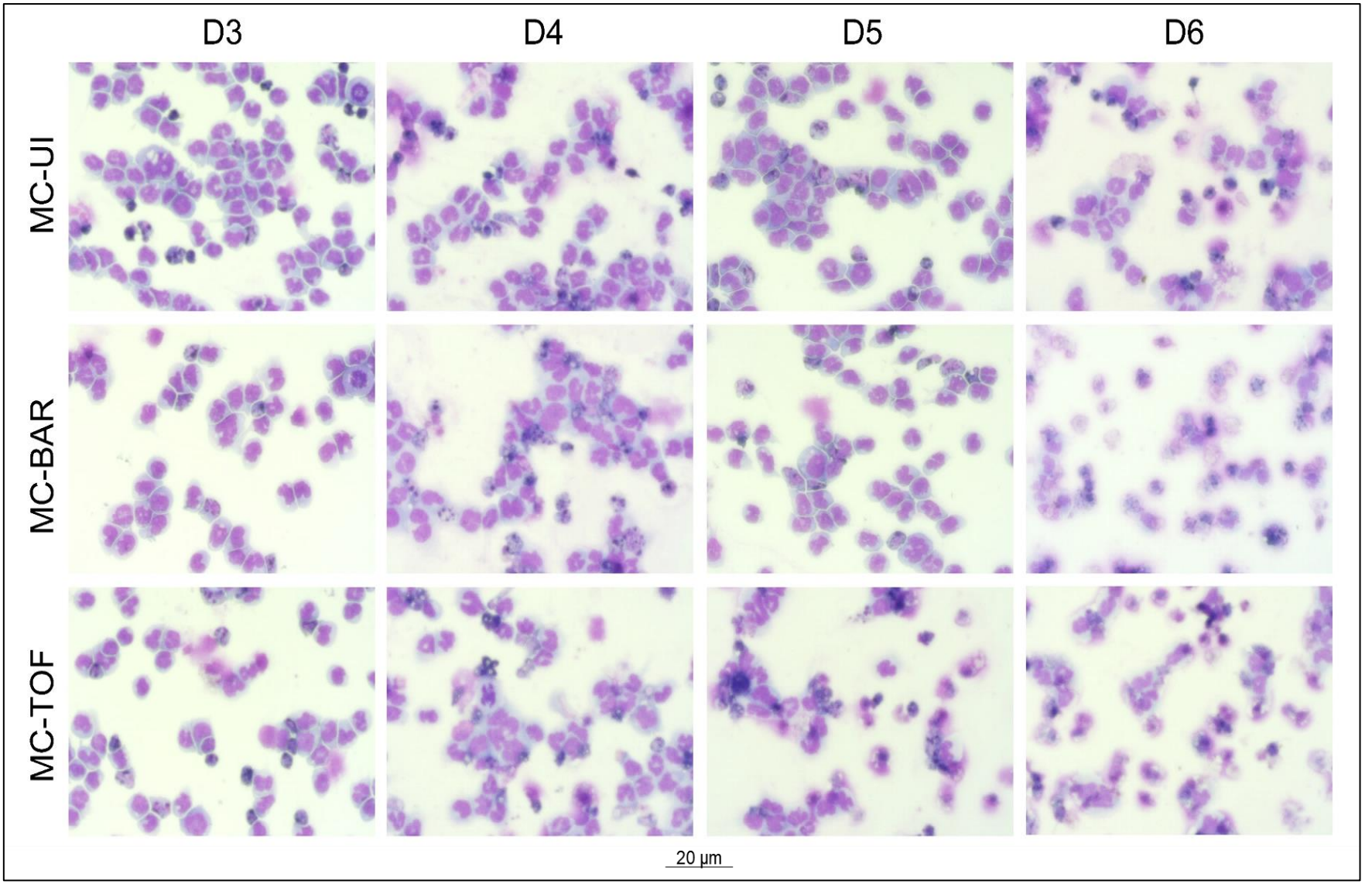

D3
D4
D5
D6
MC-UI
MC-BAR
MC-TOF
20 µm

Figure 7.9 Representative cytospin slides images of dPLB-985 cells incubated without and with JAK inhibitors. PLB-985 cells were incubated without (UI) and with JAK inhibitors baricitinib (BAR, 200ng/mL) or tofacitinib (TOF, 200ng/mL) in differentiation media and for 6 days. Fresh media changes and replenishment of inhibitors and cytokines were made on days 2 and 4. Cytospin slides of cells were prepared, stained with rapid Romanowsky stains on days 3-6, and the images captured using a microscope-mounted camera (×40).

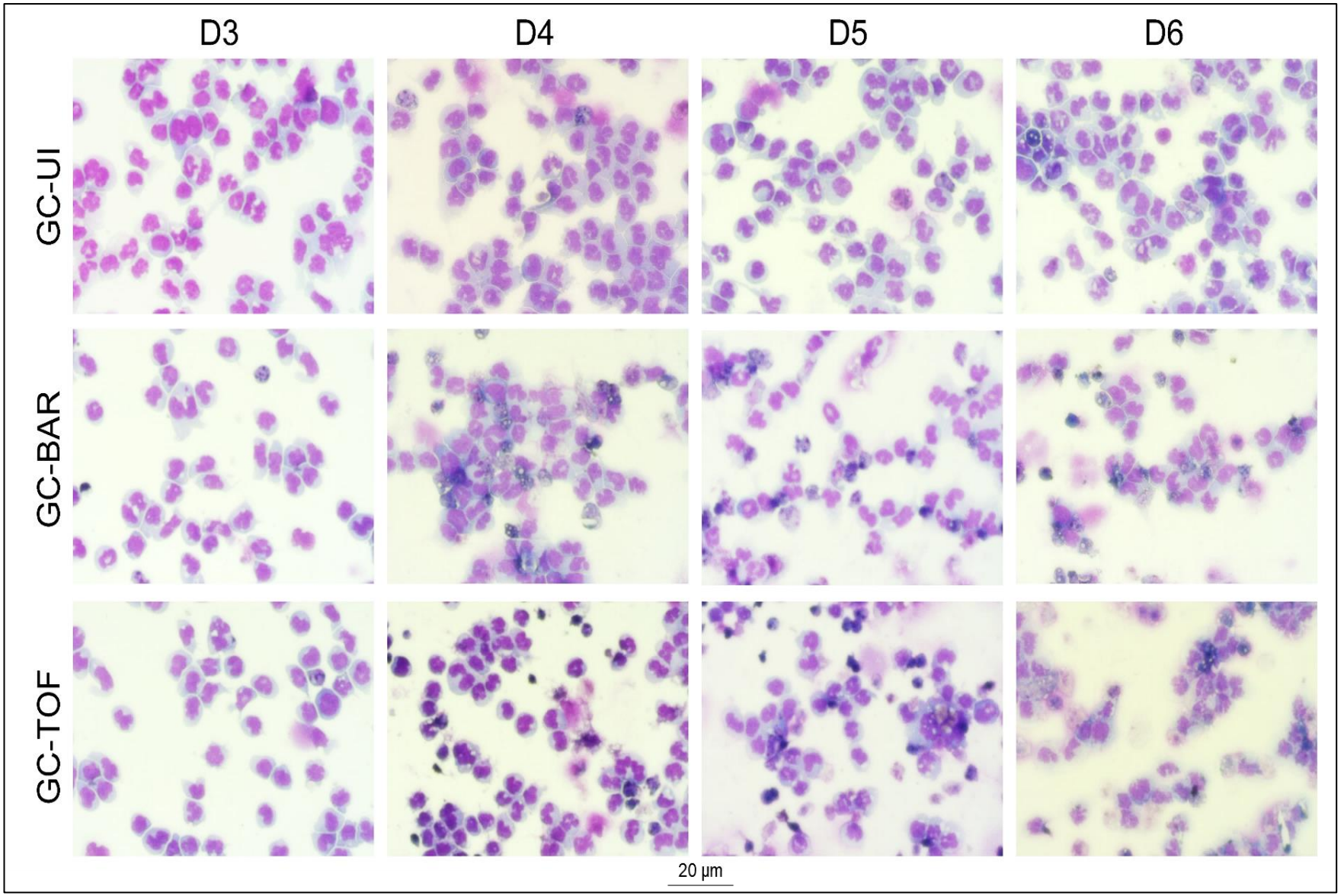

D3
D4
D5
D6
GC-UI
GC-BAR
GC-TOF
20 µm

Figure 7.10 Representative cytospin slides images of dPLB-985 cells incubated with JAK inhibitors and G-CSF. dPLB-985 cells were incubated without (UI) and with JAK inhibitors baricitinib (BAR, 200ng/mL) or tofacitinib (TOF, 200ng/mL) for 30 min, followed by the addition of the G-CSF (10ng/mL) for 6 days. Fresh media changes and replenishment of inhibitors and cytokines were done on days 2 and 4. Cytospin slides of cells were prepared, stained with rapid Romanowsky stain on days 3-6, and the images captured using a microscope-mounted camera (×40).

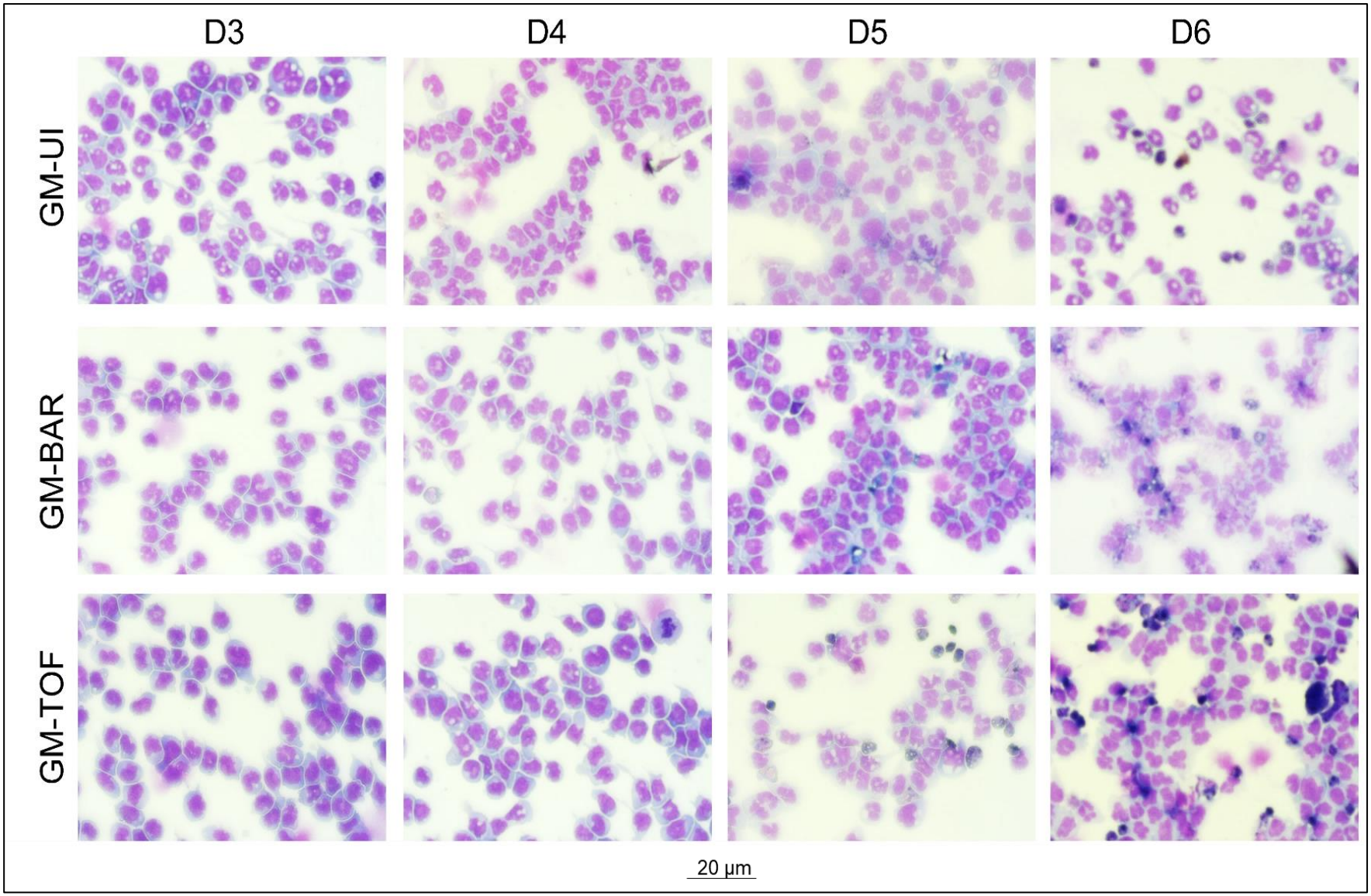

D3
D4
D5
D6
GM-UI
GM-BAR
GM-TOF
20 µm

Figure 7.11 Representative cytospin slides images of dPLB-985 cells incubated with JAK inhibitors and GM-CSF. dPLB-985 cells were incubated without (UI) and with JAK inhibitors baricitinib (BAR, 200ng/mL) or tofacitinib (TOF, 200ng/mL) for 30 min, followed by the addition of the GM-CSF (5ng/mL) for 6 days. Fresh differentiation media changes and replenishment of inhibitors and cytokines were done on days 2 and 4. Cytospin slides of cells were prepared, stained with rapid Romanowsky stain on days 3-6, and the images captured using a microscope-mounted camera (×40).

7.4 Discussion and conclusions

Dysregulation of the JAK-STAT pathway can give rise to imbalances between pro- and anti-inflammatory cytokine production by immune cells, which are implicated in the pathogenesis of autoimmunity and chronic inflammation (McInnes & Schett, 2011). The development of cell-permeable, selective, small molecule JAK inhibitors has therefore been a major advance in both understanding the molecular pathology and use for the therapy of haematopoietic, autoimmune and inflammatory disorders, in the past two decades (Furqan et al., 2013). Inhibition of JAK-dependent STATs phosphorylation, may down-regulate the activation and proliferation of key immune cells, thereby producing immunosuppressive and anti-inflammatory effects (Shi et al., 2014).

Results shown in Chapters 3 and 4 of this thesis have demonstrated that PLB-985 cells cultured in the absence and presence of differentiation inducers, undergo a limited number of divisions followed by growth arrest and apoptosis, and that the differentiation-induced cells had longer population doubling time (~24-36 h) and lower population density than their non-induced counterparts. Those initial experiments have also shown that the proliferation, differentiation capacity, and viability of the differentiated PLB-985 cells, were enhanced when the differentiation media was replaced with fresh media during culture, and these were even further enhanced when the media was supplemented with the pro-inflammatory cytokines, G-CSF and GM-CSF. These cytokines can also delay progression of the differentiated cells into apoptosis and death.

The experiments in this Chapter have examined the effects of orally-active, low molecular weight JAK inhibitors, baricitinib and tofacitinib on the proliferation, differentiation capacity and viability of differentiation-induced PLB-985 cells, as well

as on the protective effects of culture media replacements and cytokines additions, against apoptosis. Results of experiments involving differentiation media changes alone, indicated that both JAK inhibitors suppressed the growth rate, percent differentiation and viability of differentiated PLB-985 cells, cultured in unchanged media compared to the uninhibited cells. There were enhancements in these functions following differentiation media changes (at days 2 and 4), but these were also greatly down-regulated by the JAK inhibitors baricitinib and tofacitinib compared to the uninhibited (control) cells of the same culture. Therefore, these inhibitors have stimulated growth arrest and lowered the population densities of dPLB-985 cells under the two culture conditions.

The proliferation and differentiation of precursor cells committed to neutrophilic-granulocytic lineage is regulated by colony stimulating factors (CSFs) (Shimoda et al., 1997). In agreement with this, incubations with cytokines G-CSF and GM-CSF significantly increased the growth rate, percent differentiation and viability of uninhibited dPLB-985 cells compared to cultures involving media changes alone. Following incubation with both JAK inhibitors, however, these effects were also significantly decreased compared to the uninhibited (control) cells in the presence of the cytokines. This suggests that both CSFs stimulated the *in vitro* proliferation and differentiation of PLB-985 cells and equally, both JAK inhibitors down-regulated the effects of these cytokines.

Conversely, there was increasing percent apoptosis of uninhibited (control) cells cultured in unchanged media during the culture period, and this was considerably decreased following differentiation media changes. However, inhibition with baricitinib and tofacitinib, markedly increased the percent apoptotic cells, indicating that both JAK inhibitors induced apoptosis and death, in cultures with and without media changes. In a similar manner, percent apoptosis of uninhibited (control), cytokine-

incubated cells was low compared to cultures with media changes alone (no cytokine). However, in the presence of the JAK inhibitors, apoptosis increased significantly, which also implies that inhibitors, baricitinib and tofacitinib induced the differentiated cells to undergo apoptosis and abrogated the effect of cytokines in delaying apoptosis. In all these experiments, GM-CSF was more effective than G-CSF in delaying apoptosis and tofacitinib had stronger inhibitory effect on the differentiating cells, compared to baricitinib.

The four members of Janus-associated tyrosine kinases are activated differentially in response to various cytokines. JAK1 is essential for G-CSF mediated tyrosine receptor phosphorylation. G-CSF has also been shown to stimulate the activation of JAK2 and TYK2 in the absence of JAK1, but it fails to induce tyrosine receptor activation, suggesting that JAK2 and TYK2 plays only redundant or auxiliary functions (Shimoda et al., 1997). Parganas *et al*., reported JAK2 activation along with JAK1 and TYK2 in response to G-CSF, with JAK2 activation being the most significant (Parganas et al., 1998). JAK2 is the predominant JAK activated in response to a wide array of cytokines including IL-3, IL-5 and GM-CSF. Haematopoietic growth factors including G-CSF and GM-CSF and other cytokines signal through JAK2 (Quintas-Cardama & Verstovsek, 2013; Meyer et al., 2010). JAK2 deficient cells have been reported to be unable to respond to hormone-like cytokines, such as GM-CSF, thrombopoietin and erythropoietin (Parganas et al., 1998).

JAK3 plays an important, nonredundant role in the signalling of the cytokine family group that functions through the γ_c chain (Parganas et al., 1998). It associates specifically with the common gamma chain (γ_c) subunit of six cytokine receptors (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) that have critical roles in lymphopoiesis and homeostasis (Vijayakrishnan et al., 2011). The absence of JAK3 in humans has been associated with severe combined immunodeficiency (SCID) (Macchi et al., 1995).

JAK inhibitors are amongst the successful therapeutic agents that have reached clinical application (Passamonti et al., 2012). Baricitinib, a novel and potent JAK inhibitor selectively inhibits JAK1 and JAK2 enzymes with IC_{50} values in the nanomolar range (Shi et al., 2014). It has been shown to be effective in the treatment of arthritis, without adverse hematologic effects or suppression of humoral immunity (Fridman et al., 2010).

Tofacitinib on the other hand, associates exclusively with the common gamma chain (γ_c) subunit and has been shown to block signalling by JAK3-dependent cytokine receptors, which are required for lymphocyte proliferation and functions. It also blocks other cytokine receptors signalling through JAK1 (Yarilina et al., 2012; Ghoreschi et al., 2011). Tofacitinib interferes with Th1 and Th2 differentiation as well as inflammatory Th17 cells production. Recently, *in vivo* and *in vitro* studies by Tanaka and Maeshima demonstrated that tofacitinib acts on $CD4^+$ T cells to inhibit cell proliferation and inflammatory cytokines production (Tanaka and Maeshima, 2012). In addition, Ghoreschi *et al*., suggested that tofacitinib can target the cytokine signalling cascades of innate immunity, *in vivo* (Ghoreschi et al., 2011), and it has anti-inflammatory and neutrophil reduction activities (Meyer et al., 2010).

In conclusion, results of this preliminary study with baricitinib and tofacitinib demonstrated that both JAK inhibitors can suppress the proliferation, differentiation and viability of the differentiated PLB-985 cells, and they may therefore, inhibit the *in vitro* granulopoiesis. These JAK inhibitors also down-regulated the responses of the differentiated PLB-985 cells to cytokines G-CSF and GM-CSF signalling, suggesting that they could have a negative impact on granulopoiesis *in vivo* and/or perhaps down-regulate cytokine-regulated neutrophil apoptosis, effects which could be anti-inflammatory.

Chapter 8: General discussion and conclusions

Neutrophils are highly potent, motile phagocytic cells that are critical to the functions of the innate immune system. The relatively high abundance of these cells in the bloodstream highlights their importance. Approximately, 5×10^{10} neutrophils are produced in the bone marrow, in the adult human, per day, accounting for 40-65% of the total white blood cells population. This number rises significantly during infection (Edwards, 1994). Owing to their large phagocytic capacity, neutrophils destroy a wide variety of pathogens with an array of cytotoxic mechanisms, such as release of multiple degradative enzymes and reactive oxidants, without dependence on any single cytotoxic mechanism (Segal, 2005).

Mobilisation of circulating neutrophils to the site of inflammation occurs very rapidly, and constitutes a major event in the neutrophil-mediated inflammatory responses. They are recruited through a series of controlled, exocytic events, including priming, rolling and adhesion, transmigration/chemotaxis that culminates in opsono-phagocytosis and respiratory burst generation. These events change their functional status, converting them from 'resting' circulating cells to 'active' killing cells at the infection and/or inflammatory locus (Borregaard & Cowland, 1997). Neutrophils are the first leukocytes recruited to the inflamed tissue, they recognise, destroy and clear the pathogen. Following clearance of infection or inflammation, the effete neutrophils undergo a safe and non-pathological process of cell death, i.e. apoptosis and their apoptotic corpses are subsequently eliminated in a controlled manner by macrophages (Lagasse & Weissman, 1994).

Due to their high potency, cytotoxic contents and non-specific nature, neutrophils may also contribute to the pathophysiology of many autoimmune and inflammatory

diseases, such as rheumatoid arthritis, where non-specifically activated neutrophils accumulate in the joints and release reactive oxygen species (ROS) and granule proteins into the extracellular milieu, causing localised tissue damage (Cross et al., 2005), and in chronic obstructive pulmonary disease (COPD), where neutrophils undergoing necrosis (thought to be induced by *H. influenza*) infiltrate tissues and result in the tissue damage (Naylor et al., 2007). Effective control of neutrophil mobilisation and functions is therefore, imperative to prevent their inappropriate activation and impending tissue damage or persistent inflammation. This is largely achieved by a two-step activation process, and auto-regulatory mechanisms, including constitutive apoptosis.

Considering the foregoing, a clear understanding of the molecular signalling processes of neutrophils in health and disease is thus necessary. Neutrophils circulate the blood in a mature, terminally differentiated form. Hence, they lack proliferation capacity, have a short life-span, and spontaneously die by apoptosis. Furthermore, human neutrophils are currently impossible to transfect to express exogenous genes or proteins. These limitations have made their experimental manipulation *ex vivo*, challenging, and have hindered biochemical studies of their signal activation mechanisms and genetic makeup.

Establishment of a human neutrophil model, which exhibits morphological and functional properties similar to mature blood neutrophils would therefore, be very important tool in transfection studies to enable the manipulation of key genes to express modified versions of regulatory proteins and determine the effects of such modulations on neutrophil functional responses. Immunological studies involving genetically manipulated mice have shown them to be valid model system for human biology, and have continued to provide vital information on human immunology. However, there exist significant differences between human and mouse immune

systems, and many aspects of human immunology do not occur or cannot be modelled in mice (Haley, 2003; Monaco, 2003). For example, the balance of neutrophils and lymphocytes in the blood between human and mice adults are significantly different. Human blood is rich in neutrophils (50-70% neutrophils, 30-50% lymphocytes), whilst mouse blood is rich in lymphocytes (50-70% lymphocytes, 30-50% neutrophils) (Doeing et al., 2003). The functional consequences of this shift of neutrophil-rich blood in humans are therefore, not clear. In addition, many receptors expressed on human neutrophils are either not expressed at all, or are expressed differently on mouse neutrophils (Mestas & Hughes, 2004). Hence, the development of human myeloid cell lines, capable of *in vitro* differentiation, into mature neutrophil-like granulocytes, remain the most relevant model for genetic studies of human neutrophil and addresses the challenges posed by the limitations of blood neutrophils.

The first demonstration of *in vitro* differentiation of a leukaemia cell line into mature phenotype was in 1971, when Friend induced a murine erythroid leukaemia cell line to differentiate into orthochromic erythroblasts *in vitro,* using dimethylsulfoxide (DMSO) (reviewed in Watanabe et al., 1988). Following this, several other cell lines with the capacity to differentiate along the myeloid lineage into mature neutrophil-like cells in response to a wide range of inducing and maturation agents have been established. The more recently-established human promyelocytic leukaemia PLB-985 cell line, has been demonstrated to show greater levels of differentiation and was described as the current 'best' model of neutrophil differentiation (Hauert et al, 2002, Tucker et al., 1987). Previous reports have shown that PLB-985 cell line has the potential to differentiate into neutrophil-like phenotypes under the influence of various chemical agents (Tucker et al., 1987; Katschinski et al., 1999; Hazan-Eitan et al., 2006; Kim & Seoh, 2015). However, the efficiency of differentiation reported was very low, and the differentiated cells only partly, resembled mature blood neutrophils.

Therefore, the main aim of this research was to terminally differentiate PLB-985 cell line into neutrophil-like phenotypes that resemble mature blood neutrophils mostly, in particular, with respect to the typical multi-lobular nucleus and granulated cytoplasm, which are the defining features of mature neutrophils. However, terminally differentiated neutrophils undergo spontaneous apoptosis and so differentiated cells also rapidly undergo this process. A second aim was to determine ways to extend the lifespan of the differentiated PLB-985 cells. The third aim was to characterise the molecular and functional properties of the differentiated PLB-985 cells, to establish their usefulness as model of neutrophil differentiation and fuctions. The *in vitro* differentiation protocol described in this study has enabled the consistent production of morphologically- and functionally-neutrophil-like, differentiated PLB-985 cells, within periods of 4-7 days, with peak numbers occurring between days 5-6.

The main findings of the study are:

1. A modified differentiation protocol and optimisation procedures using the differentiation and maturation-inducing agents; ATRA, DMF and sodium pyruvate has been established that produced terminally differentiated, neutrophil-like PLB-985 cells that resemble mature blood neutrophil's morphology, evident by acquisition of the multi-lobed nucleus and granulated cytoplasm.
2. The cytokines G-CSF and GM-CSF, which are pro-survival for human neutrophils, enhance the growth rate, differentiation efficiency and viability of differentiated PLB-985 cells, with GM-CSF being more effective than G-CSF.
3. Terminally-differentiated PLB-985 cells undergo cell cycle arrest and progression into apoptosis and they resembled mature blood neutrophils in terms of cell cycle parameters and apoptotic morphology.

4. Differentiation of PLB-985 cells induced expression of anti-apoptotic proteins; Mcl-1 and Bcl-X_L, and their expression levels correlated with the cell's survival status, whilst Bcl-2 expression expression was lost following differentiation.
5. Differentiated PLB-985 cells undergo phagocytosis and oxidative burst activity, in response to various particles and stimuli, and in a similar manner, but with lower responses than blood neutrophils.
6. Differentiated PLB-985 cells express cell surface receptors for CD11b, CD14 and CD16. CD11b expression was high, and similar to mature neutrophils, whilst expression of CD14 and CD16 were very low in contrast to neutrophils.
7. Differentiated PLB-985 cells express strong chemotactic transmigration towards casein and activated serum, like neutrophils, but their response to fMLP and IL-8, is lower than in neutrophils.
8. Small molecule JAK inhibitors; baricitinib and tofacitinib can suppress the proliferation, differentiation and viability of the differentiated PLB-985 cells, increase their progression into apoptosis, and down-regulate their responses to G-CSF and GM-CSF signalling. They may therefore, inhibit *in vitro* granulopoiesis, and may have an anti-inflammatory activity.

Table 8.1 Morphological and functional properties of neutrophils and dPLB-985 cells.

Property	**Neutrophils**	**dPLB-985 cells**
Morphology		
Segmented, Multi-lobed nucleus	+++	+++
Granulated cytoplasm	+++	+++
Cell cycle and Nuclear DNA		
G1 arrest	+++	++
S-phase ↓	--	++
G2-phase ↓	--	+
G0 accumulation (18 h)	+++	+++
Phagocytosis		
SAPI	+++	++
F-beads	+++	++
NF-beads	++	+
Oxidative burst activity		
PMA-induced	+++	++
fMLP-induced	+++	--
Chemotaxis		
fMLP	+++	+
IL-8	++	++
Zymosan ctivated serum (C5a)	++	++
Casein	+++	+++
Receptor expression		
CD11b	+++	++
CD14	+	+
CD16b	++	--
Apoptosis		
Apoptotic features	+++	+++
Apoptotic proteins		
Mcl-1	+++	+++
Bcl-X_L	--	+++
Bcl-2 ↓	--	+++
Bax	+++	+++
Bak	+++	+++

+++ = strong, **++** = moderate, **+** = low, **--** = no activity/expression

In Chapter 3 of this thesis, PLB-985 cells were induced to terminally differentiate into neutrophil-like granulocytes that resembled mature blood neutrophils, with multi-lobed nucleus and granulated cytoplasm. These cells also stained with eosin and methylene blue to produce purple nuclei and pale cytoplasm, like blood neutrophils (Horobin, 2011; Horobin & Walter, 1989). Myeloid cells are categorized as immature; comprising blasts, promyelocytes and promonocytes or as mature; comprising myelocytes, metamyelocytes, bands and segmented neutrophils, according to the standard morphologic/staining criteria (Chomienne et al., 1990). The non-differentiated PLB-985 cells are characterised as being at the promyelocytic stage, but the combined effects of ATRA, DMF and sodium pyruvate successfully transformed them along the myeloid pathway into myelocytes, metamyelocytes, band neutrophils and lastly, segmented neutrophils, the final cells in the myeloid lineage. After 3-4 days of differentiation induction, metamyelocytes and band neutrophils were the predominant cells in the culture medium, while after 5-6 days in culture, segmented neutrophils predominated. This pattern of differentiation is analogous to that of neutrophil differentiation and maturation during *in vivo* granulopoiesis (Iwasaki & Akashi, 2007, Lieber et al., 2004). The differentiated PLB-985 cells exhibited other morphological features of mature neutrophils, including small cell size (~ 10-12 µm), indented, convoluted and segmented nuclei, decreased number or absence of nucleoli and granulated cytoplasm (Collins et al., 1978). To the best of my knowledge, this high level of differentiation and survival of the differentiated cells has been obtained for the first time in this thesis.

There was an increase in the proportion of cells showing morphological features of apoptosis, such as condensation of chromatin and fragmentation of nuclei (Elmore, 2007) in parallel with increase in the proportion of cells showing mature neutrophil morphology. Interestingly, these apoptotic cells appeared in appreciable numbers only when differentiated cells with mature neutrophil morphology predominated in the

culture, usually after day 5-7. Apoptosis is constitutively activated as neutrophils age in culture, *in vitro* (Edwards et al., 2004). In contrast, PLB-985 cells cultured in routine media without the differentiation-inducing agents remained in their promyelocytic form, exhibiting large rounded nuclei containing 2-4 nucleoli each, with a dispersed chromatin, agranulatar cytoplasm and relatively high nuclear/cytoplasmic ratio. Only very few of these non-differentiated cells displayed any sign of apoptosis. Thus, the differentiated PLB-985 cells acquired one of the most definitive features of mature neutrophils, i.e. rapid progression into apoptosis.

Another significant finding in this study was the importance of the cytokines G-CSF and GM-CSF in promoting both the efficiency of differentiation and the survival of the differentiated cells. The growth rate, differentiation capacity and viability of differentiated PLB-985 cells were enhanced when the differentiation media was periodically replaced with fresh media, but more greatly enhanced when the media was supplemented with the pro-inflammatory cytokines, G-CSF and GM-CSF. These cytokines also delayed the progression of differentiated cells into apoptosis (Thomas et al., 2010, Derouet et al., 2004, Edwards et al., 2004). The experimental approach described in this thesis, involving the combination of three differentiation- and maturation-agents, periodic medium replacements and cytokine supplementation, is unique from previously published differentiation systems. Again, to the best of my knowledge, these culture modifications and resulting enhancement of differentiation and survival of the differentiated cells have not been reported.

The changes in cell cycle parameters, progression into apoptosis and expression of key proteins that regulate apoptosis in differentiated PLB-985 cells, measured in Chapter 4 have shown that differentiation of PLB-985 cells triggers G1 arrest with little or no cells in G0 initially, but subsequently after days 3-6 of culture, there was a significantly increased number of cells in G0 phase and fewer cells in S phase. This

arrest of differentiated PLB-985 cells in G1 following differentiation into granulocytes means that the differentiated cells do not progress to the G1-to-S transition (Yen et al., 1985). G2/M arrest was also observed, suggesting that differentiation inhibited these cells from entry into mitosis, since this checkpoint is activated upon blockage of DNA synthesis and/or prevention of segregation of damaged or incompletely synthesized DNA (Pucci et al., 2000). These findings indicate that differentiation of PLB-985 cells triggers cell cycle arrests and progression into apoptosis.

DNA distribution profiles of freshly-isolated blood neutrophils revealed that most of the cells were arrested in G1 phase, with little or none in G0 (Figure 4.9). Mature blood neutrophils are terminally differentiated cells that do not replicate their DNA or proliferate. Following a 20 h incubation however, a significant number of cells accumulated in the G0 phase, indicating apoptosis and/or death. These distribution patterns are similar to those observed in the differentiated PLB-985 cells at day 6, i.e. decrease in S phase and increase in G0. This observation, further confirms that terminally-differentiated PLB-985 cells acquired some of the properties of blood neutrophils in respect to their cell cycle DNA distribution patterns.

The expression of anti- and pro-apoptotic proteins of the Bcl-2 family was investigated to determine whether changes in their expression levels may parallel and correlate with differentiation and apoptosis. This revealed that differentiation of PLB-985 cells was accompanied by a progressive decrease in the expression levels of the anti-apoptotic proteins, Mcl-1 and Bcl-$X_{L,}$ suggesting that Mcl-1 and Bcl-X_L levels correlated with the survival of the differentiated cells. Transfection studies have confirmed the role of Mcl-1 as an anti-apoptotic protein whose overexpression results in prolonged survival of cells (Reynolds et al., 1994), and plays a crucial role in the apoptosis of human blood neutrophils (Edwards et al., 2004, Moulding et al., 1998). As previously stated, Mcl-1 is the only *anti-apoptotic* Bcl-2 protein family member

expressed in human neutrophils, that has been measured reliably and reproducibly at both the protein and mRNA levels (Edwards et al., 2004).

Another striking finding was that contrary to human neutrophils, which do not express detectable levels of Bcl-X_L protein by immunoblots (Edwards et al., 2004, Moulding et al., 1998), differentiated PLB-985 cells expressed detectable levels of this protein, and like Mcl-1, levels decreased as the cells differentiate. Although, Bcl-X_L mRNA has been detected in human neutrophils, they do not express Bcl-X_L protein (Moulding et al., 2001). In the presence of GM-CSF, and in line with the ability of this cytokine to delay apoptosis, levels of these two proteins, were increased above levels observed in the absence of the cytokine. The increased levels of these two proteins parallels the delayed apoptosis in differentiated PLB-985 cells. GM-CSF has been shown to considerably delay apoptosis of neutrophils, by maintaining Mcl-1 levels (Derouet et al., 2004, Moulding et al., 1998).

Mcl-1 is an unusual member of the Bcl-2 protein family. Alignment algorithms have identified sequence similarities of Mcl-1 to other Bcl-2 family members, but the protein has some unusual properties not shared by other members of the Bcl-2 family. Its large N-terminal domain contains many motifs that determines many of its unique properties, such as subcellular localization, rate of turnover and phosphorylation status. These post-translational modifications provide Mcl-1 protein with the ability to respond to environmental signals rapidly and reversibly, and to switch the cell's fate from survival to apoptosis and *vice versa*. Mcl-1 contains 3 putative BH domains (Bcl-2 and Bcl-X_L possess 4 each) as revealed by the sequence analysis (Lutz, 2000). It is also a larger protein (40 KDa) of 350 residues, with a long N-terminal region compared to Bcl-2 (22 KDa) and Bcl-X_L (26 KDa) with 239 and 233 residues, respectively. The N-terminal region of Mcl-1 contains potential regulatory motifs, which were predicted to regulate its function and later confirmed experimentally after

mutagenesis, whereas the C-terminal region has a transmembrane domain, which is responsible for its membrane insertion and sub-cellular localization (Akgul et al., 2000).

Non-differentiated PLB-985 cells grown in routine culture media express the anti-apoptotic protein Bcl-2, but another interesting finding was that, expression of Bcl-2 was rapidly lost following initiation of differentiation after day 3 of culture, acquiring yet another characteristic feature of mature blood neutrophils that do not express Bcl-2 protein (Edwards et al., 2004). Okaro *et al*. reported that inhibition of apoptosis by Mcl-1 and Bcl-X_L, but not, Bcl-2 was responsible for the prolonged survival of both normal and tumorigenic cells in the biliary tree (Okaro et al., 2001).

The *pro-apoptotic* proteins, Bax and Bak were expressed highly in differentiated PLB-985 cells and their levels did not significantly change upon treatment with the apoptosis-delaying agent, GM-CSF. This was in line with observations in mature blood neutrophils which constitutively express a range of *pro-apoptotic* members of the Bcl-2 family proteins, including the Bax and Bak, which may partly explain the ability of neutrophils to undergo apoptosis spontaneously and rapidly (Edwards et al., 2004). Taken together, these results confirm that differentiated PLB-985 cells share important similarities with isolated blood neutrophils, in terms of cell cycle progression and apoptosis, as well as suggest an interconnection between the processes of cell cycle and apoptosis in myeloid cells.

Next, my study measured the phagocytosis and oxidative burst activity of human neutrophils and the changes in differentiated PLB-985 cells during these two vital processes of pathogen destruction and elimination. The flow cytometry data presented in Chapter 5 shows that neutrophils highly phagocytosed the opsonised PI-labeled *S. aureus* (SAPI) and opsonised fluorescent latex beads, and to a lower

extent, opsonised non-fluorescent latex beads. Priming of neutrophils by G-CSF or GM-CSF significantly enhanced the levels of phagocytosis of these particles. Similarly, differentiated PLB-985 cells showed increasing levels of phagocytosis of SAPI and latex beads in parallel with the increasing levels of their differentiation into neutrophil-like cells. The levels of phagocytosis of these particles by differentiated PLB-985 increased when primed by G-CSF or GM-CSF. However, in contrast to neutrophils, differentiated PLB-985 cells displayed higher phagocytosis of fluorescent beads, than SAPI and non-fluorescent beads.

Neutrophil phagocytosis is mediated by plasma membrane receptors which must recognise and bind the foreign bodies, either directly via pattern-recognition receptors (PAMPs) or indirectly via opsonic receptors, before internalisation and subsequent formation of a membrane-bound vacoule, the phagosome (Flannagan et al., 2012). Neutrophils express receptors for molecules such as chemoattractants (e.g., fMLP, C5a, IL-8) and opsonins (e.g. IgG, complement proteins) that facilitate recognition, binding and rapid uptake of the opsonised particles (Naccache, 2013). The observed differences in the responses of neutrophils and differentiated PLB-985 cells to SAPI are likely due to differences in the expression of receptors between the two cell-types that mediate recognition of these opsonised particles. These surface receptors may not be expressed or are expressed at different levels on the differentiated PLB-985 cells. Like blood neutrophils, differentiated PLB-985 cells took up different numbers of fluorescent bead particles, ranging from 1 to 6 resulting in multi-phasic fluorescent distribution patterns. The distribution of differentiated PLB-985 cells that took up 1, 2, 3, 4, 5 and 6 bead particles was 6%, 12%, 5%, 3% and 1% respectively, i.e. most cells took up 1-3 particles per cell.

Analysis of oxidative burst activities by flow cytometry showed that neutrophils express strong and weak responses when stimulated by PMA and fMLP, respectively.

Priming by G-CSF or GM-CSF increased the oxidative burst generation stimulated by PMA and fMLP, but this increase was particularly, greater in fMLP stimulated cells. The PMA response reaches a peak value more rapidly in primed cells. This clearly demonstrated the relevance of priming to receptor-mediated response of fMLP. Latex beads showed lower oxidative burst activity which also increased significantly when primed by G-CSF or GM-CSF. Differentiated PLB-985 cells showed very similar oxidative burst activity to blood neutrophils. High and low activities were observed in differentiated cells stimulated with PMA and fMLP respectively, and both responses increased when the differentiated cells were incubated with G-CSF or GM-CSF. Latex beads showed lowest activity which increased slightly when incubated with G-CSF or GM-CSF.

Non-differentiated PLB-985 cells are incapable of oxidative burst generation, as they produced extremely low levels of ROS with fMLP, PMA or latex beads, that did not change significantly, following incubation with the cytokines G-CSF or GM-CSF. The very low levels of ROS observed in non-differentiated PLB-985 cells could be the result of mitochondrial respiration, indicating that changes associated with differentiation of PLB-985 cells into neutrophil-like cells were responsible for their ability to elicit NADPH-dependent oxidative burst activity.

The oxidative burst activity was also measured using luminol-enhanced chemiluminescence assay, and the results revealed interesting consistency with the flow cytometry data, as well as striking similarity between neutrophils and the differentiated PLB-985 cells. Neutrophils express high and low chemiluminescence responses when stimulated by PMA and fMLP, respectively, whereas incubation with GM-CSF increased the fMLP- but not, the PMA- induced chemiluminescence. Again, this is not surprising, because fMLP binds to receptors normally on granule membranes and therefore, requires priming to mobilise the granules to the cell

surface. PMA being membrane permeable, readily enters the cell and activates PKC directly without the need for priming, though, neutrophil responses to PMA can be accelerated by priming (DeChatelet et al., 1976). Activation of the NADPH oxidase by PMA is independent of receptor binding or generation of second messages (Edwards, et al., 1990). The bacterial peptide fMLP activates neutrophils via the formyl peptide receptors (FPR) that may be present on the granules, and priming is required to mobilise these granules to the cell surface (Hallett & Lloyds, 1995). Normally, *in vivo* neutrophil activation occurs by receptor-coupled mechanisms, which is regulated by pre-exposure to priming agents, such as GM-CSF that enhance their functional responsiveness (Edwards et al., 1990).

In contrast to blood neutrophils, differentiated PLB-985 cells did not produce any chemiluminescence in response to fMLP stimulation which may be another indication of receptor expression deficiency. On the other hand, differentiated PLB-985 cells stimulated with PMA showed a single peak response similar to that of blood neutrophils. Flow cytometry measures ROS generation by NADPH-oxidase activity only, whilst chemiluminescence assay measures both the oxidase activity and myeloperoxidase ROS activities. This may partly, explain why fMLP produced ROS with flow cytometry, but not with luminol-enhanced chemiluminescence assay. Non-differentiated PLB-985 cells did not produce chemiluminescence in response to PMA or fMLP stimulation (see Figure 5.19).

fMLP stimulation produced a bimodal chemiluminescence response peak (see Figure 5.17), the first peak occurring within 2-3 min and was considered a result of extracellular oxidant generation, while the second peak occurs around 5-7 min after addition of stimulus and was considered a result of intracellular oxidants generation, probably due to entrance of luminol into the cells and its oxidation intracellularly (Briheim, et al., 1984). In contrast, PMA stimulation produces a single response peak

reaching maximum at around 5-7 min. Most of the PMA induced chemiluminescence was largely due to intracellular oxidants (Dahlgren, 1987). These observations further indicated that differentiation of PLB-985 cells was indeed, required for ROS production, measured by both techniques.

Chapter 6 of this thesis focused on the expression of some cell surface receptors, chemotaxis and activation of intracellular signalling pathways in neutrophils and differentiated PLB-985 cells, some of which mediated the functional responses identified in Chapters 4 and 5. Many of the essential components of the cytotoxic machinery of neutrophils are present in the membranes of mobilizable intracellular granules which are recruited to the plasma membrane following stimulation and degranulation (Sengelov, et al., 1993).

Results from this study indicate that both neutrophils and differentiated PLB-985 cells expressed high levels of CD11b, though expression levels on neutrophils were higher than on differentiated PLB-985 cells, and both increased following treatment with cytokines G-CSF and GM-CSF. CD11b is one of the most abundant β_2-integrins on neutrophils and is essential for their adherence to the endothelium during chemotaxis, diapedesis and phagocytosis of opsonised particles (Futosi et al., 2013). CD11b receptors are stored intracellularly in neutrophil granules from where they are recruited to replenish the cell surface levels, following stimulation with inflammatory mediators or during phagocytosis (Edwards, 1994). The mobilization of intracellular stores of some proteins, such as the adhesion molecules, is one of the underlying mechanisms for the generation of a ‘primed’ cell phenotype, which enhances the readiness of neutrophils to respond to subsequent stimuli (Volk et al., 2011).

The expression level of the cell surface receptor, CD14 was low in both neutrophils and differentiated PLB-985 cells. CD14 is known to be strongly expressed on

monocytes and macrophages, and present at lower expression levels on granulocytes (Simmons, et al., 1989). However, consistent with other reports (Fleck & Nahm, 2005; Wright et al., 1991), enhanced expression of CD14 was observed following priming with the cytokines G-CSF and GM-CSF. The level of CD14 expression on neutrophils was higher than on differentiated PLB-985 cells, with and without cytokines. Surface CD14 recognises LPS-opsonised particles and LPS-binding protein (LBP), and mediates the formation of the LPS-LBP complex, thereby facilitating the killing and clearance of Gram-negative bacteria (Vosbeck et al., 1990). In addition, CD14 binding of LPS leads to increased activity of the adhesion receptor CD11b/CD18, promoting the neutrophil response to bacterial endotoxin, LPS (Dahinden et al., 1983). CD14 has also been shown to interact with the surface of apoptotic cells, in a receptor-ligand manner, facilitating their safe elimination (Devitt et al., 1998).

Expression levels of low affinity immunoglobulin G receptor, FcγRIIIb (CD16b) showed that it was strongly expressed on neutrophils, which was further increased following treatment with G-CSF or GM-CSF. On the contrary, differentiated PLB-985 cells did not express appreciable amounts of surface CD16b, though incubation with G-CSF and GM-CSF caused an increase in its expression on these cells, the levels were nevertheless, very low compared to blood neutrophils. Stimulation of neutrophils with cytokines, such as GM-CSF triggers rapid shedding of CD16 from the surface and concurrent replenishment from internal pools (Bruhns, 2012; Perussia et al., 1983). GM-CSF maintains the expression of cell surface CD16 through mobilisation of intracellular stores of the pre-formed receptor (Moulding, et al., 1999).

A study by Selmeczy *et al.,* reported expression of CD16 on DMF-differentiated PLB-985 cells by FACS analysis (Selmeczy et al., 2003), but in contrast, Pivot-pajot *et al.*, reported a failure to detect CD16 expression on any of the PLB-985 cells

differentiated with DMSO, DMF or dbcAMP by western blot analysis (Pivot-Pajot, et al., 2010). Intracellular CD16 expression on differentiated PLB-985 cells was not detected, which could mean that there were little if any, internal stores of this receptor in the differentiated PLB-985 cells.

Neutrophils and differentiated PLB-985 cells express directional chemotactic movement towards a variety of chemoattractants. Neutrophils express stronger chemotactic responses to fMLP and casein, compared to IL-8 and zymosan-activated serum. Conversely, differentiated PLB-985 cells express strong chemotactic response towards casein and zymosan-activated serum than fMLP and IL-8. The differences in these responses of neutrophils and differentiated PLB-985 cells towards fMLP and IL-8 could be explained by the same reason as the difference observed in chemiluminescence and oxidative burst responses, described in Chapter 5, i.e., the absence or low expression of receptors by the differentiated PLB-985 cells, as these molecules function via their receptors (Futosi, et al., 2013, Bruhns, 2012, Migeotte et al., 2006). The directional transmigration of a cell towards a chemotactic attractant gradient, requires interaction of the attractant with the cell surface receptors (Van Epps et al., 1977).

Previous reports on chemotaxis of differentiated cells are inconsistent. For example, Sirak *et al.,* reported that fMLP failed to induce chemotactic migration, but stimulated the production of hydrogen peroxide in dbcAMP differentiated HL-60 cells (Sirak, et al., 1990), while Fontana *et al.,* reported that fMLP induced chemotaxis in DMF differentiated HL-60 cells (Fontana, et al., 1980). In addition, Hauert *et al*., reported that IL-8 was less effective in stimulating chemotaxis of differentiated HL-60 as compared to neutrophils (Hauert et al., 2002). Under my experimental conditions, differentiated PLB-985 cells expressed chemotactic responses towards both fMLP and IL-8, but fewer cells transmigrated compared to blood neutrophils. Therefore, my

findings agree with some reports and disagree with others. Nevertheless, these differences might be accounted for by the differences in culture conditions and assay procedures. Overall, the results of Chapter 6 confirm that differentiated PLB-985 cells express various cell surface receptors and underwent chemotactic transmigration, in response to a variety of chemoattractants, in a similar manner to blood neutrophils.

My study finally investigated the effects of low molecular weight JAK inhibitors, baricitinib and tofacitinib on the proliferation, differentiation capacity and viability of differentiated PLB-985 cells, as well as on the response of the differentiated cells to the cytokines G-CSF and GM-CSF. The results showed that growth rate, percent differentiation and viability of differentiated PLB-985 cells were suppressed and progression into apoptosis enhanced considerably, by the two JAK inhibitors. However, tofacitinib had more inhibitory effects on the differentiated PLB-985 cells than baricitinib. These effects were observed in the absence or presence of cytokines G-CSF and GM-CSF, known to promote proliferation, differentiation, maturation, and survival of granulocytes (Derouet et al., 2004; Shimoda et al., 1997; Edwards et al., 1989).

JAK-STAT signalling enhances the expression of many inflammatory cytokines and chemokines. The four members of Janus associated tyrosine kinases; JAK1, JAK2, JAK3 and TYK2 are activated differentially, in response to various cytokines. JAK1 is essential for G-CSF mediated tyrosine receptor phosphorylation, and can be activated along with TYK2 in response to G-CSF (Shimoda et al., 1997). JAK2 is the predominant JAK activated in response to GM-CSF and a wide array of cytokines including IL-3, IL-5 (Quintas-Cardama & Verstovsek, 2013; Meyer et al., 2010). JAK3 associates specifically with the common gamma chain (γ_c) subunit of six cytokine receptors (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21) that are required for lymphocyte proliferation and functions (Vijayakrishnan et al., 2011; Parganas et al., 1998).

Therefore, JAK inhibitors may inhibit production of cytokines and chemokines (Hu & Ivashkiv, 2009), and may have anti-inflammatory effects, which partly explains their efficacy in the treatment of inflammatory diseases (Yarilina et al., 2012). JAK inhibitors are amongst the successful therapeutic agents that have reached clinical application (Passamonti et al., 2012). Baricitinib and tofacitinib are among the several JAK inhibitors in advanced stages of clinical trials, for the treatment of haematopoietic disorders as well as autoimmune and inflammatory diseases (Miossec, 2013; Meyer et al., 2010).

Baricitinib, a potent and selective inhibitor of JAK1 and JAK2 enzymes, has been shown to be effective in the treatment of arthritis without suppression of humoral immunity or adverse hematologic effects (Shi et al., 2014; Fridman et al., 2010). Tofacitinib, a novel and specific inhibitor of JAK3 and JAK1, associates exclusively with the common gamma chain (γ_c) subunit to block signalling by JAK3-dependent cytokine receptors or other cytokine receptors signalling through JAK1 (Yarilina et al., 2012; Ghoreschi et al., 2011). The findings of the JAK inhibition study in Chapter 7, indicated that both baricitinib and tofacitinib can suppress the proliferation, differentiation and viability of the differentiated PLB-985 cells, facilitate their progression into apoptosis, and down-regulate the responses of the differentiated PLB-985 cells to G-CSF and GM-CSF signalling. Therefore, they may inhibit *in vitro* granulopoiesis, and have anti-inflammatory activity.

In conclusion, my study has confirmed, and extended the findings of previous research on PLB-985 cells differentiation into mature neutrophil-like phenotypes. The differentiated PLB-985 cells generated in this thesis resembled blood neutrophils morphologically and functionally, and may therefore, provide an excellent model system of neutrophil differentiation and function. Although the differentiated cells are asynchronous and had some limitations, the functional consequences of my findings

are far reaching; for example, the differentiated PLB-985 cells proved effective in studying the neutrophil properties and functions, such as apoptosis, chemotaxis, phagocytosis, oxidative burst activity and expression of cell surface receptors. Hence, these cells can be modified genetically in transfection studies to study targeted genes, and effects of their modulation on functions, in combination with primary blood neutrophils. This could facilitate understanding of neutrophil functions in health and diseases, such as apoptosis regulation in autoimmune and inflammatory diseases, as well as in neutropenia associated with bone marrow transplantation, and/or in cancers.

Future directions

While the results shown in this thesis have advanced the usefulness of the PLB-985 cell line as a model for mature neutrophils, further work is necessary to fully define the properties of the differentiated cells, and also to resolve some of the unexpected findings of the data obtained in this study. For example, a fuller characterisation of the properties of differentiated PLB-985 cells and comparison of these with mature blood neutrophils is necessary to confirm the molecular similarities and identify any components/processes that are different. This could be achieved in several ways, for example by extending the range of functions measured in the differentiated PLB-985 cells, and one key set of parameters would be identification of the intracellular signalling pathways that control blood neutrophil function. These would include measurement of expression/activation of pathways such as STAT3, STAT5, Akt, Erk and p38-MAPK in the differentiated PLB-985 cells. These signalling pathways are known to play key roles in the regulation of key neutrophil functions such as chemotaxis, respiratory burst activation and apoptosis.

One unexpected result in this thesis that would need to be explored further is the fact that while the differentiated PLB-985 cells expressed a number of surface receptors normally expressed on mature neutrophils, they only expressed very low levels of CD16b (normally expressed on neutrophils at very high levels) and they did not generate reactive oxidants or undergo chemotaxis after stimulation by fMLP. It is unknown why these receptors were not expressed. Therefore, future experiments should focus on modification of the differentiation protocol to determine if expression of these archetypical receptors can be stimulated. Additionally, it would be of great interest to compare the transcriptomes of differentiated PLB-985 cells and human neutrophils, perhaps by qPCR, a technique in routine use in this laboratory. This approach would identify and quantify the primary transcripts (mRNA), and would be a powerful way to "fingerprint" the similarities and differences between the two cell types, if these transcripts were expressed but not translated into functional proteins, then this would indicate that the differentiation programme had been correctly triggered, but other signals were required to translate the transcripts.

Furthermore, transfection studies involving genetic manipulations to knock out or knock in specific genes using plasmids or other vectors, such as by RNA interface (Brazas & Hagstrom, 2005) (RNAi) to introduce small inhibitory RNA (siRNA) that can disrupt key genes (e.g. MCL-1 and BCL-X_L) and identify changes in the expression of the Mcl-1 and Bcl-X_L proteins in the differentiated PLB-985 cells would help in gaining further insights into the neutrophil signalling pathways during apoptosis. This would also help in the validation of the differentiated PLB-985 cells as neutrophil models, as transfection of primary blood neutrophils is currently not feasible.

In addition, further studies would benefit from experimental approaches that could synchronise the differentiation of PLB-985 cells into mature neutrophil-like cells, to

generate a uniform population of cells for functional analysis. This can be achieved by changing the culture conditions to temporarily stop cell growth, such as by inhibition of growth with an inhibitor of DNA replication (e.g. thymidine, hydroxyurea or aphidicolin) and removing it later (Darzynkiewicz et al., 2011), or by removing essential nutrient(s) from the culture and re-introducing it later. In either case, cell growth can be stopped and upon release from these blockades, cells would start to re-grow and differentiate at the same stage of the cell cycle. The synchronously differentiated cells may be kept ‘viable’ for longer periods when using the cytokines G-CSF or GM-CSF, as described in this thesis.

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